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FINAL REPORT

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CHEMICAL STUDIES OF STRATEGIES FOR OVERCOMING BOVINE COPPER DEFICIENCY

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"Chemical Studies of Strategies for Overcoming Bovine Copper Deficiency"

Final Report

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NON-TECHNICAL SUMMARY

This project is the latest in a series designed to examine the underlying, chemical, causes of bovine copper deficiency, a problem in many parts of this province. This deficiency is caused not by insufficient copper in the diet, but by the presence of **thiomolybdates (TMs)**, which "tie up" the copper so that it cannot be utilised by the animal. TMs are formed from sulfur, usually in well water, and molybdenum compounds in the feed.

We have previously developed a computer "simulation" of the mix of copper species in the normal (TM-free) rumen. This has now been extended to allow for predictions involving copper administered in different forms - complexed to different amino acids, or to EDTA, for instance. We can add copper in all these forms and predict the effect on the copper species present, including the effect of a different distribution among the Volatile Fatty Acids (mimicking different feed regimens), and different ruminal pH. We have checked the validity of these simulations using Nuclear Magnetic Resonance (NMR) spectroscopy of both simulated and authentic bovine rumen liquor.

The precise way in which TMs "tie up" copper is poorly understood; there is not even agreement on the proportions in which they combine. Nor did we understand the rates at which the TMs formed. We have now developed methods for analyzing mixtures of the different TMs - this is important since different TMs differ dramatically in how they react with copper - and we have also completed the first comprehensive study of the rates at which the TMs form.

Studies on the structure of copper-thiomolybdate compounds have been somewhat hampered by delays in the commissioning of the Canadian Light Source, where much of the data was to have been collected. However, elemental analysis and other spectroscopic measurements confirm that this is a complicated, multi-stage reaction. It involves at least two stages - an initial reaction in solution, followed by a polymerization to give the final, insoluble product. There is some evidence that some at least of the copper is in a different oxidation state, and possibly also the molybdenum as well.

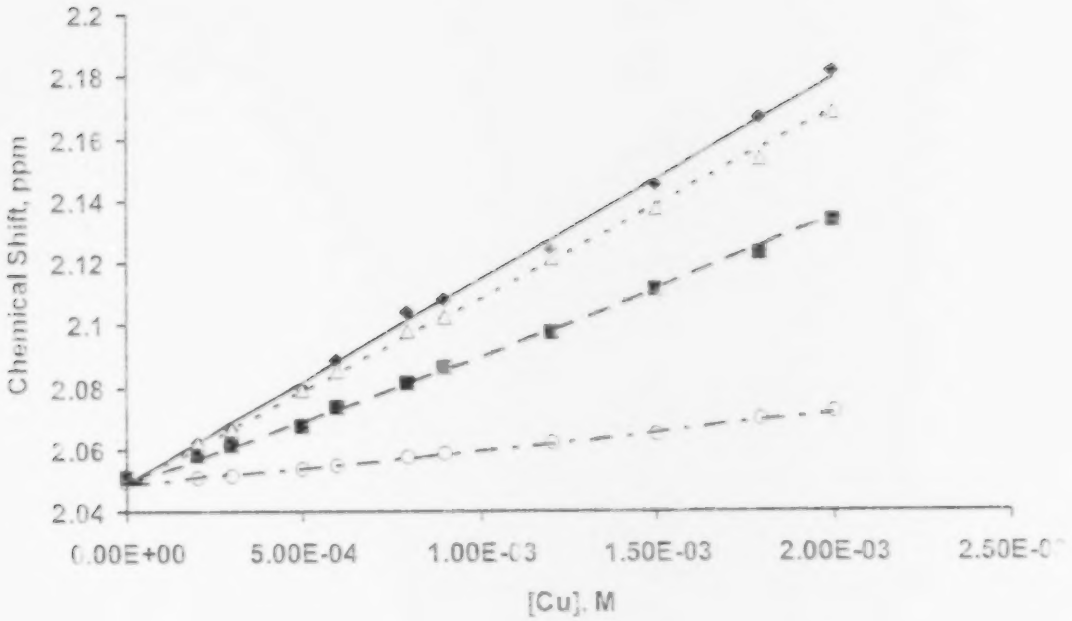
The major accomplishment of this project is the integration of the TM and TM-copper reactions into the simulation. This is technically difficult because the copper-amino acid-VFA chemistry occurs very rapidly and the TM reactions quite slowly, and they would usually be simulated by totally different methods. The model will require further refinement before it can simulate copper in the TM-contaminated rumen; this is underway in our current ADF study.

TECHNICAL REPORT

At the start of this project, we had developed the modeling of copper in the *non*-thiomolybdate (TM) - contaminated rumen to a high degree of sophistication. For instance, previous ADF projects demonstrated the lack of any real advantage to many higher-priced 'chelated' copper supplements such as Cu-Lysine, since these would decompose shortly after administration. We were able to back up these conclusions by performing actual analysis of rumen content samples by nuclear magnetic resonance (NMR) spectroscopy.

Some additional work has been done in this area. We can now incorporate additional ligands, including common amino acids used in supplements, and Histidine-containing peptides (see below). As before, we validated results using observation of the ^{1}H NMR signal from the acetate methyl protons. (The precise frequency of this signal depends upon its frequency in various forms (free in solution, complexed to copper, protonated, etc.) - which is known - and the fraction of acetate in each form - which can be predicted

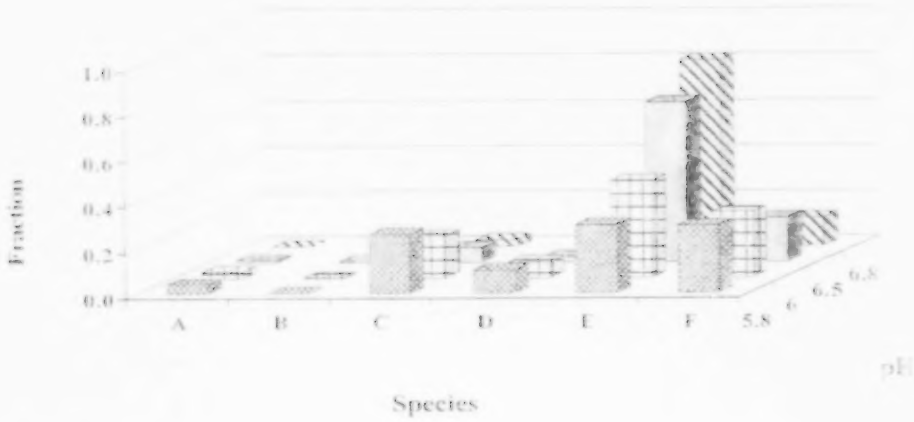
from the speciation model. The fit to experimental data is excellent, leading to high confidence in the validity of the underlying speciation model.



Changes in chemical shift of methyl protons of acetate with different amounts of (a) glycine and (b) methionine in rumen fluid as a function of Cu(II) concentration.

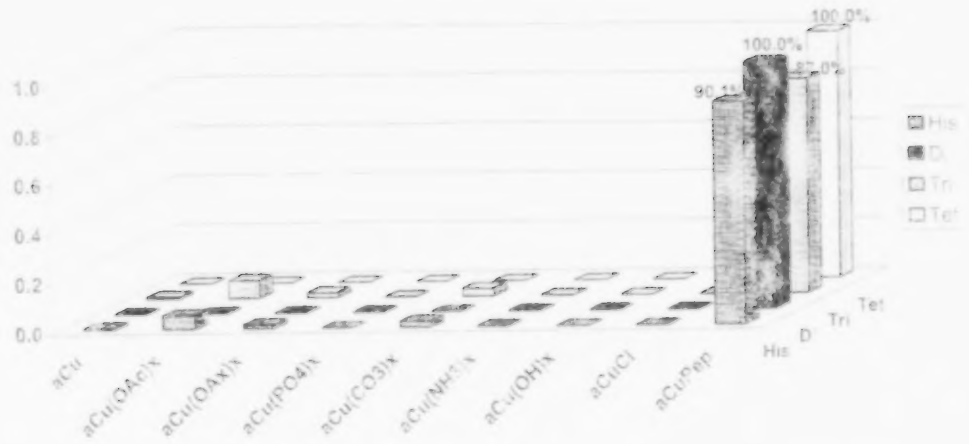
Points are experimental data, and curves are model predictions.

Using the model, it is possible to predict copper speciation in a wide variety of scenarios. Some examples are shown below.



Distribution of copper with respect to the LMM ligands in rumen fluids as pH is varied between 5.8 and 6.8. Calculations were performed at an ionic strength of 0.15 M and Cu^{2+} concentration of 3.0×10^{-5} M. See Table 1 for concentrations of ligands. **A:** Copper ammonia complexes; **B:** Free Cu^{2+}; **C:** Copper acetate complexes; **D:** Copper VFA complexes; **E:** Copper carbonate complexes; **F:** Copper phosphate complexes.

In particular, results from computer modeling suggest that the incorporation of Histidine peptides into the model may have a substantial effect on copper speciation.



A comparison of 1:1 Cu-Peptide Speciation in Rumen Fluid at a pH of 6.3.

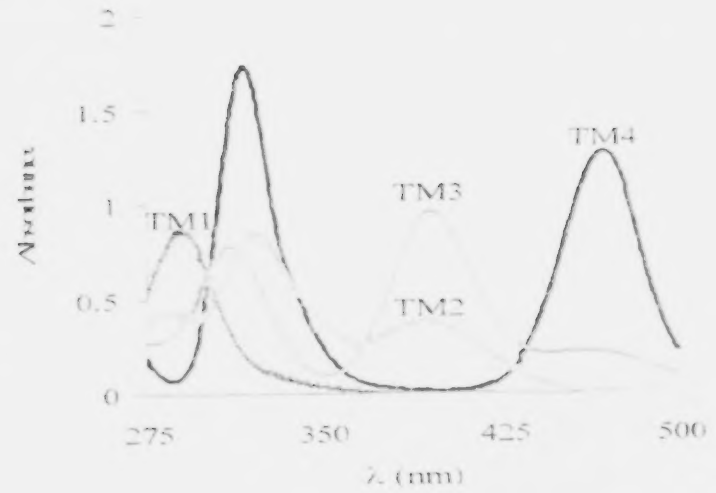
It should be emphasized, however, that the binding of copper to the peptide, although obviously strong enough to result in most of the copper being peptide-bound, is not strong enough to protect against Cu-TM formation, which we have already shown to have higher binding constants still.

Several factors make extending modeling to include TMs a major challenge. We will discuss each in turn.

TM purity. There are four TMs - with one, two, three or four sulfur atoms replacing the oxygen atoms of molybdate to give TM1, TM2, TM3 or TM4 respectively - and these are suspected to differ markedly in their degree of copper antagonism. When TMs are formed, mixtures invariably result. Using impure TM samples has resulted in decades of confusing and contradictory chemical and feed studies.

$$TM_{n-1} \underset{OH^-}{\overset{HS^-}{\rightleftharpoons}} TM_n \underset{OH^-}{\overset{HS^-}{\rightleftharpoons}} TM_{n+1}$$

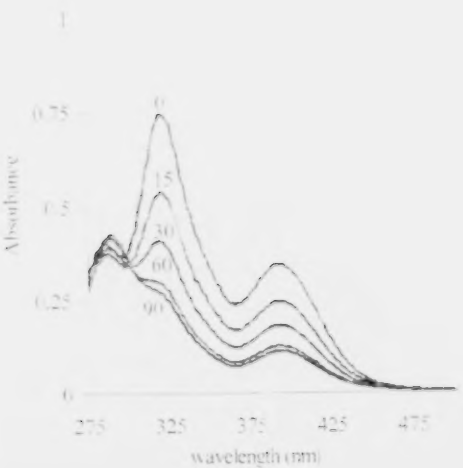
During this project, Mr. Clark has perfected an analysis to assess TM purity. The analysis of UV spectra for the analysis of impure TM samples, originally developed by Dr. E. Quagraine in a previous ADF project, allowed only for the possibility of contamination of a given TM by its "closest relatives". For instance, TM3 could be considered to be contaminated only with TM4 or TM2. However, preliminary analysis showed that in practice, we had to allow for the possibility of contamination of any TM by any other. In particular, in situations (such as the rumen!) where the TMs are being synthesized, it is quite possible that all five (including molybdate, with no sulfur atoms) are present. The UV spectra of the TMs are shown below. Note that they overlap heavily, which made generalising the Quagraine approach a substantial programming challenge.



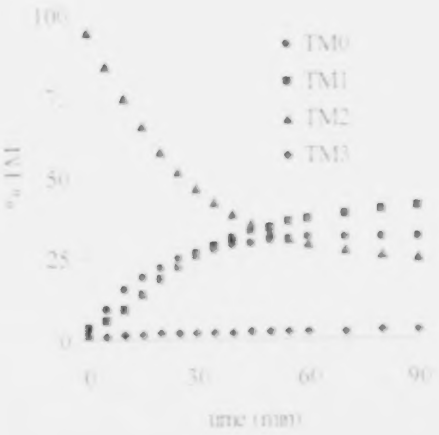
Simulated UV-visible spectra of 10^{-4} M solutions of TM1, TM2, and TM3. The TM4 spectrum is of an actual synthesized sample.

This challenge has now been met; it is possible to input a UV spectrum and have the software report a precise analysis for each TM in the sample. A recently published paper in the Canadian Journal of Chemistry, showcasing this work, is appended to this report.

TM interconversion kinetics. By doing this as a function of time, it is possible to derive a plot of the concentration of each individual TM as a function of time:



UV-vis spectra of a TM2 solution sample hydrolyzing with time. The numbers represent the incubation time in minutes.



Composition data obtained from Origin 7 fit of the UV-vis spectra shown above.

Mr. Clark has now started detailed studies of the formation and interconversion of the TMs under conditions which simulate those in the bovine rumen. The question is: of the eight possible reactions shown aoove, how many are fast enough to be of any importance, exactly how fast are they (rate constants), and how does the rate depend on reactant concentrations? Fortunately, much of the required mathematics has already been solved by Dr. Essilfie-Dughan in his studies (see below). These studies continue in our current ADF project. Once the necessary kinetic data has been determined, it will be used as input data to refine Dr. Essilfie-Dughan's rumen copper model.

The TM-copper reaction. Preliminary studies by us and others suggest that this reaction proceeded in two stages: a rapid coming together of copper and the TM to form a small, soluble complex, followed by a slower "zipping together" of those complexes to form a high-molecular weight, insoluble, polmeric material. This observation may be the key to developing 'smarter' strategies to deal with bovine copper deficiency. It is known that

copper forms a ***soluble*** complex with TMs in the presence of bovine serum albumin (BSA). This suggests that one strategy for preventing copper being "tied up" by thiolmolybdates might be to "cap off" the small, soluble Cu-TM complexes, and prevent them from polymerizing. In our previous ADF project, we reported the synthesis of small peptides (protein fragments) which mimic the part of bovine serum albumin which is thought to bind the Cu-TM complex. We were able to show that the amino-acid histidine, even in a dipeptide, could bind copper and Cu-TM. We have now modeled the speciation of copper in systems containing such peptides (see above).

As mentioned above, little is known definitively about the structure of Cu-TM adducts. Few techniques exist for studying metal ions (such as Cu and the molybdenum in TMs) in non-crystalline, polymeric materials. One such, however, is a synchrotron-based technique called Extended X-ray Absorption Fine Structure (EXAFS). The pending availability of the Canadian Light Source on campus suggested a unique competitive advantage in pursuing these studies.

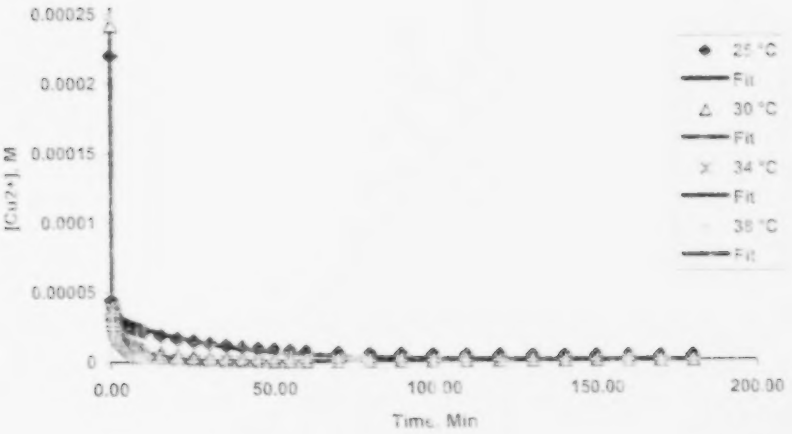
However, there were extensive delays in startup of the CLS - in fact, it was not possible to make measurements at the CLS until after the project ended. However, we have elicited the help of Dr. Graham George, a Canada Research Chair in the Department of Geological Sciences. Dr. George is a well-recognized expert in the application of synchrotron-based methods to metal speciation problems, and has even published some preliminary studies of Cu-TM materials. He has offered both to help us in winning time for Ms. Slamova's NEXAFS experiments at another synchrotron facility, and to help us

interpret the results. We are now making the intended measurements as part of our current ADF project.

Ms. Slamova has made considerable progress in the reproducible preparation of TM-Cu adducts. This is extremely exacting chemistry; we have clear evidence that redox chemistry is involved, and unless experimental conditions are very carefully controlled, different materials result every time a synthesis is performed. In a series of elegant UV-visible experiments, Ms. Slamova has established the exact stoichiometry (reacting proportions) in which the copper and TM4 or TM3 react. She has also reacted copper with TM3 and TM4 together, to establish whether the order in which the TMs encounter the copper matters (it does).

Electron Paramagnetic Resonance (EPR) spectroscopy, which detects atoms with unpaired electrons in their atomic structure, has established that the solid adducts appear to contain either Cu(I) or Mo(V), or both. Since the copper is initially Cu(II) and the molybdenum Mo(VI), something is reducing one or both of these atoms - but it is not yet clear what. It is possible to speculate about what is happening here, but a definitive answer will have to wait for the EXAFS data.

In a parallel development, Mr. Essilfie-Dughan and Mr. Clark together have investigated the use of a Copper Ion-Selective Electrode to look at Cu-TM interactions in solution.



Decrease in the concentration of Cu^{2+} versus time at pH of 4.0 and at temper-atures of 25°C, 30°C, 34°C and 38°C as Cu^{2+} reacts with TM4

Points represent experimental data and curves represent fit.

The shape of this curve is very interesting: it is ***not*** a simple exponential decay, as would be observed for a one-step process. However, it is consistent with a two-step process where the first step is reversible - further support for the hypothesis for copper-TM reaction advanced above. More detailed interpretation is difficult - the experiment only tells us that free Cu^{2+} is disappearing, not whether this is due to complexation, reduction to Cu^{+}, or possibly both.

Incorporating TMs into the rumen model. This has now been developed by Dr. Essilfie-Dughan. This work had to overcome three challenges:

1. The TM-free rumen copper model is an equilibrium model. Since copper-amino acid, copper-VFA etc. reactions are known to be rapid, one can assume that all reactions will very quickly proceed to an extent dependent on the amounts of reactants and the equilibrium constants of the various complexes - a mass-action model. However, TM interconversions are slow (~ minutes), and have to be modeled by kinetic (rate-based)

means. To treat both together, the TM kinetic scenario is allowed to "evolve" for a short time interval, then the copper equilibrium speciation rearranged at that point in time.

2. To determine rate data, or to model it, one must solve a series of differential equations. In this case, the equations involved are extraordinarily difficult (~impossible) to solve directly; however, an indirect method involving Laplace transforms allows solution of the mathematics. (For an overview of how this works, see the slides frm Dr. Essilfie-Dughan's Ph.D. seminar, appended to this report.)

3. Neither the precise kinetic model, nor the relvant rate constants, were known, as Mr. Clark had just commenced those studies. Dr. Essilfie-Dughan simply assumed a model and some preliminary rate constants (which we don't entirely trust) from the scientific literature. At time of writing, it appears that Dr. Essilfie-Dughan's assumed kinetic model is probably correct, so all that is required is to substitute Mr. Clark's definitive rate constants in the model as these become available.

PUBLICIZING RESULTS

All three students presented their results at the Canadian Society for Chemistry Annual Conferences in May, 2005, in Saskatoon, and in May, 2006 in Halifax.

Several manuscripts are in preparation, or submitted for publication. Two of particular importance have been appended to this report. The first, "Accurate UV-visible spectral analysis of thiomolybdates", was recently published in the Canadian Journal of Chemistry. The second, "Improved Speciation Modeling of Copper(II) Supplements in

Bovine Saliva and Rumen Fluid", has been submitted to the Journal of Inorganic Biochemistry.

We have also appended a set of "handouts" from Dr. Essilfie-Dughan's Ph.D. seminar to the Department of Chemistry. Although this was presented after the termination of this project, it is a good summary of this work for any reader not ready to wade through the detailed explanations of the full papers.

A copy of Dr. Essilfie-Dughan's Ph.D. thesis has been placed on file with the ADF, and can be consulted for full details. It has not been appended since it runs to more than 200 pages. Copies of the Clark and Slamova theses will be forwarded to the ADF when available (estimated Spring 2007).

POTENTIAL FOR COMMERCIALIZATION

This is fundamental research, so it is not expected that direct commercialization would usually be an issue. The ultimate goal of this work is a nutritional strategy or a compound which could protect against thiomolybdate-based copper deficiency. Such a product might well be marketable.

EXPENDITURES OVER $500

None.

PERSONNEL INVOLVED

Joseph Essilifie-Dughan's Ph.D studies form much of this project. His particular strengths are in the areas of computer modelling, and in understanding the subtle details of copper complexation processes. He has since graduated (Spring 2007) and is currently a postdoctoral fellow with Prof. J. Hendry of the Department of Geological Sciences at the University of Saskatchewan.

Rhett Clark joined the group approximately four years ago as an M.Sc. student, and has since transferred, on the basis of superior performance, to the Ph.D. program. He brings a sound knowledge of chemistry from his training at the University of Regina, and has developed useful additional skills since arriving. He specializes in the area of TM characterization and kinetic modelling.

Darina Slamova also joined the group approximately four years ago as an M.Sc. student, and has since transferred, on the basis of superior performance, to the Ph.D. program. She is an inorganic chemist by training, well-suited to tackling the subtle issues involved in Cu-TM interactions. She is pursuing the structural studies of TM-copper complexation.

STATEMENT OF EXPENDITURES

This has been forwarded under separate cover by the University of Saskatchewan. It will be noted that I was supporting three graduate students on a budget originally intended for one and a summer student! This is possible because of the students receiving substantial additional support from teaching activities within the Department. In particular, Mr. Clark and Ms. Slamova are both recipients of Graduate Teaching Fellowships because of superior teaching in undergraduate laboratories. The project was concluded within its budget.

Accurate UV–visible spectral analysis of thiomolybdates

R. Stephen Reid, Rhett J. Clark, and Emmanuel K. Quagraine

Abstract: The UV–visible spectra of various thiomolybdates have been studied. Cross-contamination during synthesis and extensive overlap in the spectra have hindered the UV–vis characterization to date. We report the use of multivariate curve fitting for the accurate quantification of the composition of thiomolybdate mixtures as well as the molar absorptivities at key wavelengths of the spectrum of each pure thiomolybdate. The synthesis and isolation of all four thiomolybdates by a common synthetic route using $(NH_4)_2S$ is also reported. This is the first time that monooxothiomolybdate has been isolated from aqueous solution.

Key words: Thiomolybdates, UV–visible spectroscopy, speciation, curve fitting.

Résumé : On a déterminé les spectres UV–visible de divers thiomolybdates. Des contaminations croisées au cours des synthèses et des recouvrements dans les spectres avaient toujours empêché leur caractérisation jusqu'à maintenant. On a fait appel à une méthode d'ajustement de courbe à plusieurs variables pour faire une quantification précise de la composition de mélanges de thiomolybdates et pour déterminer les absorptivités molaires à des longueurs d'onde clés de spectres de chacun des thiomolybdates purs. On rapporte aussi la synthèse et l'isolement de chacun des quatre thiomolybdates par une voie de synthèse commune à l'aide de $(NH_4)_2S$. C'est la première fois que le monothiomolybdate a pu être isolé à partir d'une solution aqueuse.

Mots-clés : thiomolybdates, spectroscopie UV–visible, spéciation, ajustement de courbe.

[Traduit par la Rédaction]

Introduction

Thiomolybdates ($MoS_xO_{4-x}^{2-}$, herein called TMx where x represents the number of S ligands, are known to cause copper deficiency in ruminants (1). For an overview of TM chemistry, see the review by Laurie (2). TM interconversions are relatively slow processes (minutes to hours), and the determination of these rate constants is paramount to modeling the actions of copper in the rumen. The work reported here forms part of an ongoing study toward this goal (3, 4).

TM4 and, likely, TM3 are the primary copper antagonists, while TM2 and TM1 are probably not responsible (5). However, because of the sequential nature of TM formation, the synthesis of pure (>99.5%) TM compounds is difficult. A reliable means for determining the composition (±0.1%) of all TMs in a mixture is crucial for quantitative study.

TMs are formed by successive substitution of O with S ligands (Scheme 1). The synthesis is well-known in general terms and has been performed for some time (6), with the exception of TM1, which is said to be difficult to isolate (6–8). Previously, the synthesis required H_2S gas, which is toxic and harmful to the environment. However, since H_2S has a pKa1 of 7 and a pKa2 of about 17 (9), and TM synthesis is performed at pH values ranging from 10 to 12, HS^- is certainly the dominant sulfide species and probably the nucleophilic species involved in the reaction. This was first proposed by Harmer and Sykes (6).

Scheme 1.

$$TM_{x-1} \underset{OH^-}{\overset{HS^-}{\rightleftharpoons}} TM \underset{OH^-}{\overset{HS^-}{\rightleftharpoons}} TM_{x+1}$$

To synthesize a pure TM, the reaction conditions must be such that reaction is stopped when the desired TM is at a maximum. To favor further substitution of S, one can decrease the S:Mo ratio, decrease the pH, increase the temperature, and increase the reaction time. To synthesize TM1, TM2, and TM3, careful adjustment of the parameters is necessary, and even then, it is extremely difficult to avoid cross-contamination with other TMs. An effective means of characterizing the TM purity and composition is therefore required.

Pure TM4 synthesis, on the other hand, is very easy because the reaction is simply forced to proceed to completion, avoiding cross-contamination. Manipulation of the synthesis by increasing reaction times and using greater S:Mo ratios did not effect the UV–vis spectrum. This indicated that TM4 was already being obtained at 100% purity.

TM purity has been obtained by a variety of techniques and reported in the literature. One common method used is elemental analysis (6, 10, 11). However, a positive result with elemental analysis must be backed up by another method because an equimolar mixture of the two surrounding TMs gives the same theoretical values as the pure de-

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sired TM. Also, the differences typically determined between theoretical and experimental percentages of Mo and S are insufficient for accurately determining purity.

^{95}Mo NMR has shown some promise for determining TM purity. Each TM gives a single, sharp peak, well-resolved from the others (12). Despite the nucleus's significant quadrupole moment, the electronic environment around Mo in all four thiomolybdates is apparently sufficiently symmetric to result in minimal quadrupolar broadening. ^{95}Mo, however, has a natural abundance of 15.72% and is a low-sensitivity nucleus resulting in a poor signal-to-noise ratio. To get a reasonable spectrum for quantitative analysis, high concentrations (~0.1 mol/L) and long acquisition times (>10 min) are needed. Contributions of other TMs, up to about 5%, may go undetected. TMs are also rather unstable in water with decreased stability with decreased number of S atoms. These long acquisition times mean that hydrolysis will occur before the spectrum is obtained.

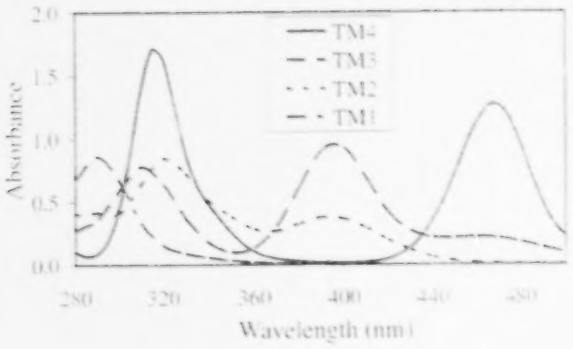
Ogra et al. used fast atom bombardment mass spectrometry (FAB-MS) to identify the presence and molecular mass of the TM of interest (13). However, attempts in our group to quantify purity using FAB and electrospray (ES)-MS were ineffective because of a large degree of fragmentation (12). This is partly due to degradation and hydrolysis of the samples at the high temperatures that were incurred. ES-MS also caused a loss of S during the analysis and may have even reacted with the less-stable TMs (TM1 and TM2) to form increased amounts of TM3 or TM4.

All TMs give characteristic UV–vis spectra (Fig. 1), making this technique useful for speciation. It has been used extensively for this purpose (6, 10, 14, 15). The strongly overlapping absorbances, however, make it difficult to identify molar-absorptivity constants because purity cannot be readily determined by inspection. Originally, elemental analysis was used to confirm a pure compound, and then the UV–vis spectrum was taken and used to directly measure absorptivity constants, which were subsequently used as a reference for purity. The uncertainty of the purity of the original compound makes the resultant absorptivities suspicious.

Some workers have used single-wavelength measurements, selecting a wavelength where there are only two absorbing species, particularly when performing kinetic studies (6, 16). The assumption made in such an approach is that contributions of all other TMs in the sample to the spectrum are negligible. The quantities of the absorbing species are determined by a simple fit. This method relies on the quality of the absorptivity constants available as the composition is directly related to it. It also requires that the assumed species make up 100% of all absorbing TM species present. This is rarely the case. Thus, it can be concluded that the monitoring of a single wavelength is insufficient for quantitative determinations.

TM1 has proven to be difficult to synthesize in pure form. The only reported isolation of TM1 was the potassium salt using methanol at –15 °C for 24 h with a yield of 10% (17). Otherwise, its presence has been inferred using UV–vis spectroscopy during TM formation (18). Supposed characteristic λ_{max} and ε (molar absorptivities) have also been inferred during reaction in solution (15) but again do not account for contamination.

Fig. 1. Simulated UV–vis spectra of 10^{-4} mol/L solutions of TM1, TM2, and TM3. The TM4 spectrum is of an actual synthesized sample.



The entire TM2 spectrum is overlapped by TM3, a common contaminant when synthesizing TM2. This makes a determination via standard Beer's Law curve highly suspected. The corresponding elemental analysis seen in the literature for TM2 also leaves doubt that the original sample was pure (10).

Because mutual TM contamination is so common, an effective method for determining accurate concentrations of individual TMs in mixed-TM samples is necessary. Preliminary results obtained by analyzing data at two wavelengths using Microsoft Excel® readily displayed discrepancies with literature results obtained from univariate analysis. This demonstrates the need for a more-robust mode of analysis.

Multivariate curve fitting provides simultaneous determination of large numbers of parameters; in this case, these parameters are molar absorptivities of all TMs present at each wavelength selected as well as the fractional composition of all species present in a given sample. This method has a number of advantages over univariate analyses. First, it eliminates the need for selecting an appropriate single wavelength. Second, absorbance errors will be averaged out over data at all wavelengths and will not directly affect any single result. It also removes the linear dependence amongst the parameters. Overall, multi-wavelength analysis is more-robust, and the determination of purity simultaneously with absorptivities improves the accuracy of both values. For an overview of mathematical modeling, see the text by Gershenfeld (19).

This paper describes the synthesis of TMs using $(NH_4)_2S$ solution as a reagent. Curve-fitting techniques are then used to determine both the absorptivities for each TM and the composition of TM mixtures in aqueous solutions. This work differs from others in the literature in that there are no assumptions made as to the initial purity of the synthesized samples. TM1 is also isolated for the first time.

Experimental

All chemicals used were reagent-grade and used without purification. Sodium molybdate ($Na_2MoO_4 \cdot H_2O$, 99.5%–103%), cesium chloride (CsCl), aq. ammonia solution (28%–30%), and anhyd. diethylether were obtained from BDH (Toronto, Canada). Ammonium sulfide (minimum

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20% $(NH_4)_2S$ and Sephadex® G-25 were obtained from Sigma (St. Louis, USA). Anhyd. ethanol was purchased from Commercial Alcohols Inc. (Brampton, Canada). The thiomolybdates: mono-, $Cs_2MoO_3S{\cdot}1/2H_2O$; di-, $(NH_4)_2MoO_2S_2{\cdot}2H_2O$; tri-, Cs_2MoOS_3; and tetra-, $(NH_4)_2MoS_4$, were synthesized as described in this paper. H_2O was millipore-grade and was deoxygenated with argon before use.

TM absorptivities

UV–vis spectra were taken on a Beckman 640 diode array spectrometer with single beam using a 1 cm quartz cuvette. Spectra were typically taken over a range of 275 to 500 nm. When measuring TM0-contaminated TM1 samples for the purpose of determining molar absorptivities, a range of 200 to 500 nm was used.

The pure salts of TM0 and TM4 were dissolved in water, and the UV–vis absorbances were measured. Absorptivities were determined from the slope of the Beer's Law plots at the desired wavelengths. TM1 and TM3 were synthesized to contain contamination from only TM0 and TM4, respectively. All TMs were added to clean, dry volumetric flasks. Measurements were taken immediately after dilution to avoid hydrolysis.

Preliminary studies showed that TM1, the least stable TM in solution, underwent minimal hydrolysis in the first three minutes. TM2 was stable for about 10 min with TM3 and TM4 showing increasing stability. Errors due to hydrolysis are expected to be minimal for TM1 and negligible for all other TMs.

The absorptivities of these TMs were then determined by curve fitting using Origin® and the principle that absorbances are additive (see eq. [1]). TM2 was synthesized and characterized in the same fashion.

$$[1] \qquad A = \sum_{i=0}^{4} \varepsilon_i c_i$$

The subscript i represents the TMs (0–4), c is the fraction of a given TM, and ε is the molar absorptivity at a given wavelength. A and c are the absorbance and concentration, respectively.

Test solutions containing all TMs were made by dissolving TMs of known composition in water with varying concentrations and measuring their UV–vis absorbance. For hydrolysis experiments, a single TM of known purity was dissolved in water and made to volume. In this case, the solution was left in the cuvette to hydrolyze and the UV–vis spectra were taken at regular time intervals. Typically, 5 min intervals were used, but in the case of TM3, hydrolysis was sufficiently slow that 10 min intervals were used. For TM4, 30 min intervals were used.

Synthesis

For TM1, TM3, and TM4, the presence of air was not found to have an effect during synthesis, and so no precautions were taken. TM2, however, was synthesized under inert atmosphere, as discoloration due to oxidation occurred quickly in air. All TMs were stored under Argon.

TM1 $(Cs)_2MoO_3S{\cdot}1/2H_2O$

$Na_2MoO_4{\cdot}H_2O$ (1.0 g, 4.13 mmol) was dissolved in a solution of water (1.2 mL) and 8 mL of aq. ammonia (28%–30%) and placed in an ice bath to reduce the temperature to ~5 °C. 2 mL of $(NH_4)_2S$ solution (20%) was also cooled in an ice bath and was added to the molybdate solution. The reaction mixture (~pH 13) was mixed and allowed to stand for 10 min before a CsCl solution (2.8 g, 16.63 mmol in 2 mL of water) was added. The reaction mixture was added to a previously cooled mixture of 15 mL diethyl ether and 5 mL ethanol, shaken, and left to stand to precipitate for about 15 min. A fine yellow precipitate (1.39 g, 75% yield) formed. This precipitate was filtered and washed three times with 10 mL of ethanol then twice with 10 mL of diethyl ether. The pale yellow, crystalline $Cs_2MoO_3S{\cdot}1/2H_2O$ was dried and stored under an inert atmosphere. The level of hydration was determined by X-ray crystallography (20).

TM2 $(NH_4)_2MoO_2S_2{\cdot}2H_2O$

$Na_2MoO_4{\cdot}H_2O$ (1.0 g, 4.13 mmol) was dissolved in 1.2 mL of water and 5 mL of aq. ammonia (28%–30%) and cooled in an ice bath. 4.8 mL of cold $(NH_4)_2S$ solution (20%) was added, mixed, and allowed to react in an ice bath for 10 min. Precipitation occurred by adding 20 mL of a cold 1:1 ethanol/ether mixture, followed by 20 mL of ether and then 12 mL of ethanol. The mixture was allowed to stand for another 15 min before the fluffy yellow-orange precipitate was filtered out. This precipitate easily browned in air, so precipitation was performed in a filter flask attached to a Schlenk line with thorough washing with ethanol then ether. The dried TM2 was quickly transferred to a storage vial and stored under inert atmosphere. The product was amorphous, making X-ray crystallography ineffective. A ^1H NMR standard-additions experiment was used to determine the stoichiometry of hydration.

TM3 Cs_2MoOS_3

$Na_2MoO_4{\cdot}H_2O$ (0.5 g, 2.07 mmol) was dissolved in 1 mL of water and 1 ml of aq. ammonia (28%–30%). To this was added 5 mL of $(NH_4)_2S$ solution (20%). To avoid precipitation of $(NH_4)_2MoO_2S_2{\cdot}2H_2O$ before the addition of CsCl, neither solution was precooled, and the mixture was mixed with gentle swirling. The mixture was then allowed to stand at room temperature for 8 min before a CsCl solution (1 g in 2 mL of water) was added. Deep-orange precipitate formed immediately, and 10 mL of 1:1 ethanol/ether was added to improve the yield (71%). The product was washed with 10 mL of cold water, twice with 10 mL of ethanol and twice with 10 mL of diethyl ether, dried, and stored under inert atmosphere. The addition of non-polar solvents to the filtrate caused the product that was dissolved by water to precipitate. This product is of higher purity as determined by techniques described elsewhere in this paper. The product was then filtered, washed with ethanol and ether, and stored under inert atmosphere.

TM4 $(NH_4)_2MoS_4$

$Na_2MoO_4{\cdot}H_2O$ (1.0 g, 4.13 mmol) was dissolved in 2 mL of water, and to it was added 15 mL of $(NH_4)_2S$ solution (20%). The contents (~pH 10) were stoppered, shaken, and allowed to stand in a 35 °C water bath. After about 3 h, each

Table 1. Molar absorptivities of the TMs at λ_{max} from this work and selected references for comparison.

	This work			Erickson 2000 (15)			Muller 1969 (21)	
	λ_{max}	ε	$\pm$	λ_{max}	ε	$\pm$	λ_{max}	ε
TM0	207	10 100	200				205	8400
							231	2800
TM1	290	8600	50	290	7390		288	8000
				314	2900		314	5000
TM2	286	4300	50	290	3940	40	288	3000
	321	8470	60	322	6740	60	318	6000
	395	3790	30	394	3230	50	394	3000
TM3	312	7330	40	312	7500	40	313	6600
	396	9550	80	396	9030	80	392	8700
	461	1860	20	462	2080	10	465	2300
TM4	317	17 300	300	318	16 480	190	317	18 000
	468	12 800	200	468	11 870	150	467	13 000

Note: λ_{max} is in nm, and ε is in L·mol^{-1}·cm^{-1}. $\pm$ in this work is the standard deviation from the experimental values; $\pm$ in reference 14 is the standard deviation of the slope of absorbance vs. concentration curves.

green rod-like crystals began to form, but the mixture was allowed to stand for at least 4 h to ensure completeness of reaction. 10 mL of a cold mixture of 1:1 ethanol/ether was added, shaken vigorously, and left in an ice bath for 30 min to improve the yield (79%). The precipitate consisted of fine dark-red crystals with a green shimmer as well as the rod-like crystals that had formed previously. The product was filtered and washed with 10 mL of cold water, 10 mL of 1:1 ethanol/ether, and 10 mL of ether before storing under inert atmosphere. As with TM3, a second precipitate formed in the filtrate. This was filtered and washed with 10 mL of 1:1 ethanol/ether and twice with 10 mL of ether to obtain a red scaly precipitate (22% yield). This product was also dried and stored under inert atmosphere.

Results and discussion

UV–vis characterization

The inevitability of mutual contamination of TM samples makes it necessary to have an effective means of analyzing the composition of the sample. UV–vis spectroscopy is a useful technique for analyzing TMs, but it is limited by the great deal of overlap in the TM spectra. It also typifies the limitations of assuming that a synthesized TM is pure when measuring its spectrum.

To use UV–vis effectively for speciation, the molar absorptivities of each pure species must be determined despite the inevitable contamination of all real samples. The Origin7 software contains a curve-fitting routine that was used to find these constants. The molar absorptivities at λ_{max} of the pure TMs as determined by fitting are shown in Table 1, along with selected literature values for comparison. Errors for TM1, TM2, and TM3 are in terms of standard deviation of the measured and fitted absorbances. For TM0 and TM4 where ε values were determined by Beer's Law plots, errors are standard deviation of the slope. Solution concentrations are assumed to be exact.

The use of curve-fitting techniques allows for the simultaneous fitting of multiple variables. The software iterates all parameters beginning at preset values. Iterations stop when the overall error, the difference between actual and fitted values, is minimized. Also, to reduce the error, a minimum of ten solutions of the same composition but differing concentrations were fit.

Curve fitting was done by fitting the absorbances at a subset of selected wavelengths. The wavelengths included all λ_{max} as well as a few other chosen wavelengths that would help to characterize all of the peaks such that all peaks were represented by at least three wavelengths. If too many points are selected, there is no further improvement of the results. If there are too few points selected, there may be more combinations of absorptivities possible to fit the data, especially when there are multiple TMs overlapping, or all 13 wavelengths were selected.

When fitting UV–vis data of the TM solutions, there are two sets of unknowns, the absorptivities of each pure TM at the selected wavelengths and the composition of the real (impure) TM sample. These unknowns cannot be fit simultaneously, and so each set was fit separately.

The absorptivities were fit using eq. [2]

$$[2] \qquad A_\lambda = \left[\sum_{TMs} a_{TM} \varepsilon_{TM,\lambda} \right] c_{TM} / Fm_{TM}$$

A_λ is the absorbance at wavelength λ, a_{TM} is the fraction of a given TM in the impure "TMx" sample, c_{TM} is the grams/L concentration of the impure TM sample, Fm_{TM} is the average formula mass of the sample, and $\varepsilon_{TM,\lambda}$ is the molar absorptivity of TMx at wavelength λ.

Absorbance values are the dependent variables, and each array consists of absorbances at an individual wavelength for

curred by all solutions. The concentrations of the solutions are the independent variables, and the absorptivities are the parameters to be fitted. The absorptivities of any known TMs and the fractional TM composition are input as constants.

The fractional TM composition is set at an estimated value based on inspection of the UV–vis spectrum of the sample. The formula mass is then calculated from the formula mass of each TM component and the fraction present in the sample. However, it must be noted that the counter ion of each TM is dependent on which TM was the target of the synthesis. For example, when synthesizing TM3, the target would be $Cs_2MoO_3S{\cdot}1/2H_2O$, and so any TM4 that forms would be Cs_2MoS_4 as opposed to the ammonium salt that would be produced by TM4 synthesis. If the contaminating TM was accompanied by a different counter ion, the FM would not be determinable prior to the fit. Figure 2*a* illustrates the quality of fit for absorptivity determination. Some wavelengths have been removed for clarity.

Attempts to determine all TM absorptivities simultaneously failed because of the large number of variables and the great dependency of the variables on each other. Each TM was therefore characterized individually. TM0 and TM4, which were available in pure form, were measured directly, and their absorptivities were determined via Beer's Law curves.

TM4 is the final product in the series to be formed. The ability to synthesize it in pure form facilitated the characterization of the other TMs. We are certain of the purity of TM4 because the reaction could be driven to completion by increasing the S:Mo ratio, a more acidic pH, and a longer reaction time. It was found that attempts to force the reaction further, i.e., even greater S:Mo ratios, resulted in no changes to the product, indicating that only TM4 was present.

Further evidence of TM4 purity can be seen in the UV–vis spectrum. It contains no evidence of a peak around 395 nm. This would be indicative of TM3, the primary contaminant. Also, when mixtures of synthesized TMs were measured, there were no irregularities observed between theoretical and actual values. Misidentified TM4 purity would have become evident at this point.

Each subsequent TM was carefully synthesized to contain contamination only by already characterized TMs. For example, synthesized "TM3" contained TM4 but no TM2, while "TM1" contained only TM0 as a contaminant. Initially, the identities of the contaminants were determined by qualitative inspection of the UV–vis spectra.

The TM3 or TM1 absorptivities were thus the only unknowns. This reduced the number of variables and improved the quality of fit. It should be noted that, initially, all TMs were included to establish those that weren't present. Once all absorptivities were known, fitting was again repeated with all TMs included to ensure the results.

The second fitting stage involved finding the composition of a given impure sample. The absorbance is still the dependent variable, but the array consists of the absorbance values of all selected wavelengths for one solution. The independent variables are now the TM absorptivities from the fit in step 1. The solution concentrations and average formula mass are input as constants, and the α values are the parameters to be determined. Figure 2*b* shows the quality of fit when determining the TM composition. Again, some wavelengths were removed for clarity.

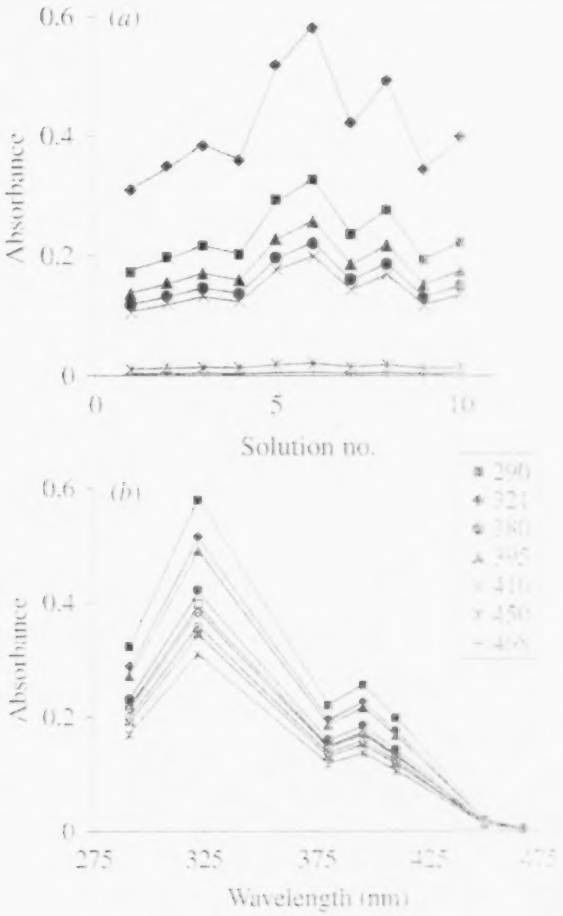


Fig 2. (*a*) Origin7 fit to find molar absorptivities. (*b*) Origin7 fit to find TM composition in impure sample. The data points are the selected wavelengths used in this study; the solid lines represent the fit. Each curve was obtained by measuring solutions of varying concentration but constant composition. The legend values are in nm.

With the TM fractions determined, the average formula mass was recalculated based on the new results, and the composition was reacquired. This process was repeated until the parameters no longer changed. The concentrations were then input into the first-fitting program to recalculate the absorptivities, which were subsequently used to redetermine the composition. This process was repeated until convergence was achieved.

There are some significant discrepancies between absorptivities achieved in this work compared with other works. This is due to the characterization method in conjunction with potentially impure samples that are assumed to be pure. By determining the composition concomitantly with the absorptivities, all values are more reliable and not dependent on the purity of the synthesized samples.

In the case of TM1, the absorptivity obtained in this work at 290 nm is greater than the absorptivities obtained from other works. Because TM1 was difficult to synthesize, this value was previously obtained by monitoring its formation in solution. During formation, there is no point at which only TM1 is present because of the rapid formation of TM1 and TM2. It is likely that TM2 will begin to form before conversion to TM1 is complete. The presence of TM2 accounts for the second small peak that was often recorded as belonging to TM1 (6, 15, 18). Being able to synthesize and isolate the TM1 salt was paramount to being able to characterize TM1 and subsequently TM2. This will also improve our ability to study TM kinetics in the future.

TM2 was difficult to synthesize in pure form. It was also very difficult to identify contamination by inspecting the UV–vis spectrum. The presence of TM1 is completely overlapped by the TM2 peak at 286 nm. Contamination by TM3 would produce an increase in the peak at 321 nm. Even though TM3 absorbs around 461 nm, the absorbance is weak, and so small amount of TM3 may go unnoticed in the TM2 spectrum. It also may be possible to have some contribution of TM0 in the sample, which does not absorb in the studied region, and would reduce the overall absorbance.

The TM3 spectrum gives away the fewest clues about contamination. One method of attempting to quantify purity was to find a characteristic peak ratio between the peaks at 396 and 461 nm (11). There has been very little agreement on this value in the literature (7, 8, 11). However, the presence of TM2 will decrease both absorbances by a similar amount, since the difference in absorptivities between the TMs at both wavelengths is similar. This method still carries the assumption of purity.

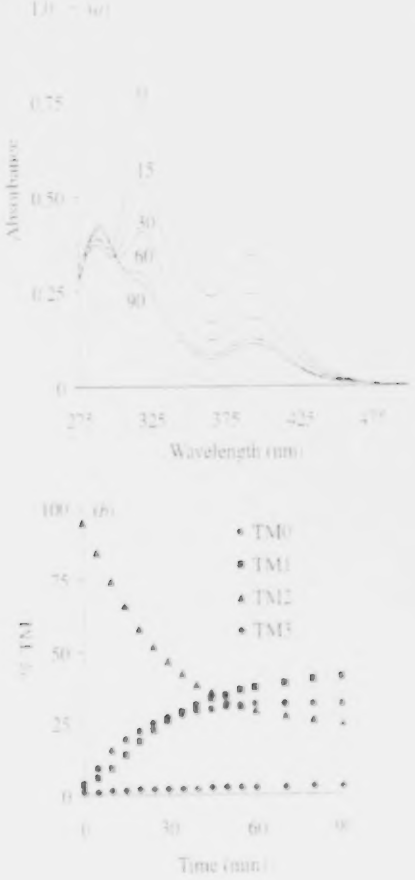
Qualitatively, one can identify the primary contaminant in synthesized "TM3". Around 461 nm, TM3 has a plateau. The formation of a discernable peak is due to TM4. If TM2 is the primary contaminant, a shoulder should appear at 286 nm. This may be difficult to observe if there is only a small amount of TM2 present. Curve fitting is a reliable way of quantifying TM impurities in TM3.

The primary difference between this method of characterization and others is that purity and absorptivities are determined simultaneously. Direct measurement of absorptivities may result in very good precision, but the accuracy is intimately dependant on the initial purity of the sample. By fitting both absorptivity and composition together, the error in one is not compounded in the determination of the other. This method also utilizes the mass of the sample and accounts for the actual formula mass and not that of the target species. This minimizes errors incurred by assuming molar concentrations.

The ultimate goal of this work is to be able to find TM concentrations in a mixture. Equation [2] was used to determine the percentage of each TM in the solution at that time. All TMs were included in the equation. Because only one solution is being fit at a time, the error in the fit is expected to be greater than is achieved from fitting multiple data sets at once. This error is much more sensitive to the quality of the original solution. However, the results over time are expected to be consistent.

Figure 3*a* shows UV–vis spectra of a "TM2" sample in water as hydrolysis occurs. For clarity, some spectra in the series have been removed. Figure 3*b* shows the percentage of each TM present during each measurement. This task was performed with all different TMs as the starting material with the same success (data not shown). These percentages can be easily converted to TM concentrations for kinetic analysis.

Fig. 3. (*a*) UV–vis spectra of a TM2 solution sample hydrolyzing with time. The numbers represent the incubation time in minutes. (*b*) Composition data obtained from Origin7 fit of UV–vis spectra in Fig. 3*a*.



The increase in absorbance at 461 nm indicates the formation of TM3 as is confirmed during fitting. This is significant because it shows that hydrolysis is not a unidirectional process. This phenomenon has implications with the kinetic treatment of the data. This will be discussed in future publications.

The rise in the peak at 286 nm is accompanied by a slight bathochromic shift, indicating an increase in TM3. TM1 absorbs more strongly than TM2 at this wavelength, resulting in an overall increase in absorbance. The strong decrease in absorbance at 321 and 396 nm indicates the loss of TM2. TM1 absorbs very weakly in these regions, and TM3 is being formed in very small quantities.

After about 90 min, there is very little change in the UV–vis spectrum. Because the reaction is occurring in a sealed

cuvette, sulfide cannot be released to the atmosphere and thus remains in the space above the solution. Equilibrium is attained between the sulfide in gas phase and liquid phase, and so the TM composition stabilizes. No TM4 is formed in this experiment, as expected.

These observations are mirrored in the fitting results shown in Fig. 3*b*. A good quality of fit was maintained throughout the analysis. The percentage TM curves also appear to have very little scatter, indicating that the fitting is sensitive to small changes in concentration.

The ability to determine TM concentrations accurately provides the basis for being able to accurately calculate TM formation and hydrolysis rate constants toward the ultimate goal of modeling rumen copper. These results are currently being pursued and will be reported at a later date.

Conclusion

Multi-wavelength curve fitting has been used to accurately characterize the UV–vis spectra of the TMs as well as to quantify TM mixtures. Effective characterization is a crucial step toward measuring the kinetics of TM interconversions and their relevance to Cu antagonism, which in turn is necessary for our final goal of modeling Cu–ligand speciation in the bovine rumen. Synthesis and isolation of all TMs are also reported using a common synthetic route using $(NH_4)_2S$ as a sulfide source. TM1 was isolated in solid form for the first time from aqueous solution.

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IMPROVED SPECIATION MODELING OF COPPER(II) SUPPLEMENTS IN BOVINE SALIVA AND RUMEN FLUID

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ABSTRACT

Copper bioavailability in ruminants is intimately related to the particular metal ion species that exist in different body compartments. In previous studies, computer simulations of copper(II)-ligand speciation in different biofluids were reported. For bovine saliva, good agreement was displayed between the results of simulations and experimental ^{1}H NMR data. However, substantial discrepancies were observed between computer simulation and experimental values for rumen fluid. The present study explores the reasons for this discrepancy, and refines the computer simulation. Good agreement is now obtained between the computer simulation and experimental values, upon introduction of copper(II) mixed–ligand species into the model. The study was extended to include glycine, histidine, methionine and EDTA complexes as the copper source in both rumen saliva (McDougall's solution) and rumen fluid. Results show that only the histidine and EDTA complexes persist to any significant extent, in spite of the large number of competing ligands present in these matrices.

KEY WORDS

Copper supplements; speciation modeling; bovine rumen.

INTRODUCTION

The importance of copper in ruminant nutrition is well established as ruminants require Cu for a number of enzymes involved in numerous functions [1]. Thus, deficiency of copper is a severe problem which leads to distinctive pathologies in ruminants and eventually results in economic loss to farmers.

The deficiency can be caused by low dietary Cu (primary deficiency) or antagonistic behavior of thiomolybdates in the rumen of ruminants (secondary deficiency) [2]. Cu deficiency in ruminants can be corrected by administering either free (inorganic) or complexed (chelated) copper. Chelated forms are thought to be more bioavailable as they are more stable, and may therefore be absorbed intact. However feed studies conducted to compare $CuSO_4$ (an "inorganic" supplement) and copper lysine (a "chelated" supplement) showed dietary Cu from the two sources to have similar bioavailability [3, 4]. This result has been substantiated in previous studies in our group, in which computer simulations and ^1H NMR were used to study the speciation of copper(II) lysine and copper(II) sulfate in McDougall's solution (simulated bovine saliva). Speciation of copper in bovine saliva was shown to be near-identical, whether the metal was introduced in inorganic form or as a lysine complex; in both instances, copper is present mainly as carbonate and phosphate complexes [5].

To fully understand copper uptake, it is necessary to identify the chemical forms in which copper will exist as it journeys through the GI tract. Thus, the computer modeling was expanded to include the major low molar mass ligands present in the rumen [6]. This study provided further evidence that complexes such as copper lysine would

disintegrate in the GI tract. The validity of this rumen model was further evaluated by ^{1}H NMR investigations on biological samples.

However, substantial discrepancies were found between the model and predicted values as additional lysine was introduced into the model, the deviation increasing as the added concentration of lysine increased. If the model correctly mimics the true behaviour of the system, it should correctly predict the observed effect of changing added ligand concentrations in the system. The inability to do so in this case therefore calls the general validity of the model into question, even in cases where agreement appears satisfactory. In the study reported here, the discrepancy between the computer modeling of copper lysine speciation and experimental results from NMR studies are investigated, and the model refined to remove the discrepancy.

For better understanding of the speciation and bioavailability of copper in ruminants, there is the need to study the speciation of other chelated copper supplements in biofluids of ruminants so as to establish a good model for copper distribution in ruminants from one body compartments to the other [7]. Thus, we have also performed computer simulations of copper glycine, copper methionine, copper lysine and Copper EDTA (chelated copper supplements) in McDougall's solution (bovine saliva). As before, ^{1}H NMR has been used to validate speciation results.

Since the reliability of the simulation model hinges on the precision and accuracy with which the complex formation constants are measured, it was necessary to examine the formation constants of the copper glycine, copper histidine and copper methionine

under rumen conditions. Use was made of formation constants determined in our group under conditions mimicking those in the rumen [8].

MATERIALS AND METHODS

Computer Modeling of Speciation

Rumen Model. The rumen model included those major end products of rumen metabolism which could serve as as potential ligands for copper(II). These are mostly the short chain volatile fatty acids (principally acetic, propanoic and butanoic), with ammonia, bicarbonate and phosphate from the saliva. It is difficult to lay down exact figures for the analytical concentrations of the various ligands in the rumen, as these are highly dependent on the diet fed to the ruminant. A set of "typical" values was assigned, after perusal of the literature [9-13]; these are shown in Table 1. The concentration of copper used for the model was 3.5×10^{-5} **M**, a modest value for the nutritional requirements of a healthy cow [14, 15].

Table 2 shows the set of formation constants used in the rumen model. The constants were selected with respect to the ionic strength and temperature in the rumen from various thermodynamic equilibrium constants databases [16, 17]. Formation constants obtained under different temperature and ionic strength conditions can also be corrected using *The Adjustment, Estimation and uses of Equilibrium reaction constants in Aqueous solution suite of programs* (Aq_solutions) *©2004* [18].

Using the values in Tables 1 and 2 as input values, computer simulations were performed to determine the distribution of copper with respect to low molecular mass ligands in the rumen fluid. Most calculations were performed at an average ruminal pH

of 6.3. However, ruminal pH can vary considerably. To judge the effect of this upon copper(II) speciation, a study was also performed with copper(II) and ligand concentrations held at typical values and the pH varying from 5.8 to 6.8. Levels of the different VFAs are also known to vary substantially depending on diet. For instance, diets high in concentrates tend to produce fluids that are high in acetic acid but low in propanoic acid, whereas forage diets are known to increase the level of propanoic acid [12]. Calculations were therefore repeated with varying concentrations of VFAs.

Copper supplements in saliva and rumen. Also using the concentration of the low molecular mass constituents of bovine saliva (McDougall's solution) and the formation constant of the different complexes [5] as input values, a simulation was done to determine the distribution of copper lysine, copper glycine, copper methionine, copper histidine and copper EDTA in McDougall's solution. The acid dissociation and copper(II) complex formation constants of the copper amino acid complexes (Tables 3 and 4) were obtained from previous studies in our lab [5, 8]. Computer simulations were also conducted to determine the distribution of copper lysine, copper glycine, copper methionine, copper histidine and copper EDTA in rumen fluid.

Simulation Calculations. The mathematical model is chosen on the basis of the known chemistry of the system, using a series of equations based on the law of mass action to define the system. These equations are then used to generate numerical values for concentrations of each species formed, based on assumed concentrations for a small number of "seed" species. Calculations were performed in a Microsoft Excel® spreadsheet running on an IBM compatible PC 1.4 GHz (Intel Pentium IV) Gateway microcomputer. The advantage of using a spreadsheet is that one can watch the species

values at each iteration. Observing progress towards convergence makes the method more understandable, and often reveals some of the subtleties involved. The sum of all species involving copper(II), or a given ligand, should add up to the total (added) concentration for that entity in all forms. The algorithm performs this summation for each entity, and compares the value obtained with the actual total concentration of that entity. The worst estimate is selected for adjustment; the corresponding "seed" value is corrected down (or up) by the factor by which the calculated total concentration overestimates (or underestimates) the true value. When convergence has apparently been attained, formation constants of all species are back-calculated and compared with the input values to check for any discrepancy. This ensures that the predicted equilibrium position is indeed fully consistent with all input data.

NMR Studies of Rumen Fluid

Chemicals. All reagents used were of analytical grade and were used as purchased without any further modifications. Ammonia solution and sodium hydrogen carbonate were obtained from British Drug House (BDH) Chemicals, Toronto; acetic acid Potassium Chloride from EM Science (Affiliate of MERCK); propanoic acid from Fischer Scientific Company, Fair Lawn, NJ, U.S.A.; butanoic acid from Aldrich Chemical Company, Milwaukee, WI, U.S.A.; sodium hydrogen phosphate and L-lysine from Sigma - Aldrich company, St Louis, MO, U.S.A.; and copper (II) chloride from Allied Chemicals, NY, U.S.A. Sodium 2,2-imethyl-2-silapentane-5-sulfonate (DSS) was obtained from Merck Sharp & Dohme (Montreal, Canada). Glycine in the

"monohydrochloride" form, and L-Histidine, were obtained from the Sigma-Aldrich Company (St. Louis, Missouri, USA). Lysine "monohydrochloride" was obtained from Advanced ChemTech (Louisville, Kentucky, USA). Methionine was obtained from Alfa Aesar (A. Johnson Matthey Company, USA). Sodium hydroxide and ethylenediaminetetraacetic acid (EDTA) were obtained from VWR International (West Chester, PA, USA)

^{1}H NMR experiments. Solution mixtures containing concentrations of ligands used in the rumen model and various copper(II) concentrations were prepared in 100 ml volumetric flasks. The ionic strength in each case was adjusted to 0.15 **M** using the required amount of potassium chloride. 10 ml aliquots of the prepared solutions were placed into labelled vials and the pH of each solution adjusted to a pH of 4.00 (± 0.01) by the addition via syringe of small amounts of concentrated solutions of KOH or HCl.

0.4 ml of each of the pH-adjusted solutions was placed into an NMR tube and 0.05 ml of a 1% solution of sodium dimethyl-2-silapentane-5-sulfonate (DSS) added as an internal chemical shift reference. The ^{1}H NMR spectrum of each sample was collected on a Bruker Avance 500 spectrometer operating at a frequency of 500.13 MHz, and the chemical shift of the methyl proton resonance of acetate recorded. The binomial 1331 pulse sequence technique for water suppression was employed. All chemical shifts are quoted in ppm versus DSS.

pH Measurements. These were made with an Accu-pHast standard combination glass body pH electrode (Fisher Catalogue No. 13-60-297) and an Fisher Accumet pH meter. The electrode was calibrated by standard U.S. National Institute of Standards and

Technology (N.I.S.T.) approved methods, using a set of primary standard solutions whose pH values have been accurately established by N.I.S.T [19].

Rumen liquor Samples. Rumen liquor samples were obtained and treated as outlined previously [5,6]. ^{1}H NMR data were collected on a Bruker Avance 500 spectrometer operating at a frequency of 500.13 MHz.

Calculation of Predicted Chemical Shifts. The fractions of the various acetate species were calculated as above. These were then input into Equation 1 (see Results Section), together with the known values of the acetate methyl proton chemical shifts [6].

RESULTS AND DISCUSSION

Computer Modeling of Cu(II) Speciation in the Rumen The results obtained by modeling of the speciation of Cu(II) in the presence of the low molecular mass (LMM) ligands of the bovine rumen environment indicate that the bulk of copper is present as carbonate. This is in spite of the relative high concentrations of volatile fatty acids (VFAs), especially acetic acid, compared to other LMM ligands in the rumen (Table 1). Figure 1 illustrates the distribution of copper with respect to the LMM ligands of rumen at the average ruminal pH of 6.3. 60.4% of the copper exists as carbonate species, 23.8% as phosphate, 10.3% as acetate, 3.8% as the other volatile acids, 1.4% as ammonia and 0.2% as aquated copper. $\alpha Cu(CO_3)_x$ represents the fraction of all copper carbonate species; $\alpha Cu(PO_4)_x$, of all copper phospate species; $\alpha Cu(NH_3)_x$, of all copper ammine species; αCu, of "free" ionic copper; $\alpha Cu(OAc)_x$, of all copper acetate species; and $\alpha Cu(OA_x)_x$, of all copper complexes of the other volatile fatty acids present in the rumen.

(see Tables 1 and 2). This distribution results from the relatively higher binding strength of carbonate to copper compared to the rest of the LMM ligands present in the rumen, as detailed in Table 2. The result obtained is similar to that obtained for copper speciation in McDougall's solution (bovine saliva) [5]; copper was mainly present as carbonate species. This implies that carbonate is able to hold on to copper right from the mouth to the rumen in spite of the myriad LMM ligands it encounters. It is therefore not surprising that copper carbonate has been used successfully as a copper supplement for ruminants [20].

Fluctuations in pH, which are common in the rumen, were also incorporated into the model. The changes that occur across the normal ruminal pH range are shown in Figure 2. As the pH increases, $\alpha Cu(OAc)_x$ falls off and $\alpha Cu(CO_3)_x$ and $\alpha Cu(PO_4)_x$ dominate up to the highest normal ruminal pH of 6.8, where copper exists mostly as carbonate species. A small fraction of the total copper concentration is attached to the rest of the volatile fatty acids – propanoic, butanoic, isobutanoic, pentanoic and isopentanoic, and a tiny fraction attached to ammonia. According to the model, copper is virtually completely bound in the rumen fluid, with only trace amounts of "free" ionic copper existing. This is in agreement with other studies that report an immeasurable amount of ionic copper in biological fluids. This can be directly attributed to the high complexing ability of the copper(II) ions.

Concentrations of VFAs in the rumen vary over a wide range, with acetic acid being dominant [9-13, 21, 22]. The observed proportions of VFAs are related to composition of the diet. Roughage diets high in cellulose give rise to acid mixtures particularly high in acetic acid. As the proportion of concentrates in the diet is increased, the proportion of

acetic acid falls and that of propanoic acid rises. Calculations performed under varying concentrations of VFAs did not result in any significant changes in the overall distribution of copper(II), as shown in Figure 3. It is known that absorption of volatile fatty acids does in fact take place in the rumen. They are absorbed across the rumen wall in the free form, apparently without active transport. Since some of complexes copper forms with VFAs are neutral, it might well be that these complexes are absorbed alongside VFAs. This will mean that some copper absorption takes place even prior to its arrival at the small intestine, where most of the copper absorption takes place. However, the extent to which copper complexes to these acids is minimal, and thus the role the copper-VFA complexes play is unlikely to be significant. We conclude that variations in the proportions of the different VFAs produced will not markedly affect copper absorption.

Computer Modeling of Speciation of Chelated Cu(II) Supplements in Bovine Saliva

Mineral supplements are usually administered orally and since the saliva is the first fluid that the metal encounters once admitted into the digestive system of the animal, it is logical that a study of copper speciation in the animal begins with the saliva. It is well known [23] that the pH of bovine saliva, in the absence of dietary acids, varies throughout the day between 5.5 and 8.0, averaging about 6.0. The speciation of Cu(II) supplements in bovine saliva was calculated over this pH range. All the calculations were done at an ionic strength of 0.15 M and temperature of 38 °C with copper introduced as 1:2 copper-amino acid complex mixtures. Results of the simulation show that whether the copper is introduced as copper(II) glycine, copper(II) methionine or copper(II) lysine makes little difference to the resulting copper speciation in

McDougall's solution. In all three cases, carbonate species predominate. This is consistent with an earlier study in our group in which the speciation of copper lysine and copper chloride in McDougall's solution were compared [5].

In all three cases, the concentration of "free" copper at all levels is found to be low, and decreases with increasing pH values. This is because of the high affinity of copper ions for coordinating ligands. The chloride, sulfate, and the hydroxide ligands do not compete effectively for the copper(II) ions in solution. Although there is a preponderance of chloride ions in saliva, it is not unusual for transition metals to avidly complex with other ligands instead. In many other biological fluids the chloride is bonded only after all the bidentate and polydentate ligands have been complexed [24].

When the copper is introduced as copper(II) histidine and copper(II) EDTA in McDougall's solution, the predicted speciation in both cases suggests that about 90% of the copper will be retained as copper histidine and almost 100% of the copper is retained in the form of copper EDTA complexes over the entire physiological range when copper is introduced as copper histidine and copper EDTA respectively. A comparison of the copper speciation in McDougall's solution at pH 7.0 for the copper supplements discussed above is shown in Figure 4. Similar results was obtained for the copper speciation in simulated rumen fluid (Figure 5).

Our present understanding is that copper(II) histidine and copper(II) EDTA are the supplements that remain intact in as they traverse the GI tract from the mouth to the rumen. This is not surprising; at pH 7, copper(II) complexes of all four amino acids (glycine, methionine, lysine and histidine) exist as bis complexes (CuL_2) [8], as shown

in Figure 6. Glycine, methionine and lysine bond to copper(II) through their carboxylate oxygen and the nitrogen of the amino nitrogen, but histidine bonds through its amino and imidazole nitrogens. Since the copper(II) ion is classified as a borderline hard acid, it has a binding preference for nitrogen- rather than oxygen-based ligands. Thus, histidine has a higher copper(II) complex formation constants (see Table 4) and hence is able better to hold onto copper(II) than glycine, methionine and lysine in the presence of other competing ligands.

EDTA's strong binding to copper (see Table 4) can be attributed to the chelate effect: it uses of its four carboxylate oxygens and amine nitrogens for bonding, as shown in Figure 7.

Validation of Computer Model with ^1H NMR Experiments Computer simulations of speciation in complex biological matrices are necessarily incomplete; they include only only those parts of the system that the researcher considers relevant. It is therefore crucial to obtain some assurance that the model is a realistic (if simplified) representation of the true system, and will yield reliable predictions under all conditions likely to be encountered. Such testing involves using the model to predict the value of some parameter which can also be measured experimentally.

As we have previously reported [6], the ^1H NMR chemical shift of the acetate methyl protons responds in a sensitive manner to changes in the extent of complexation by Cu(II). Using the speciation model, it is possible to predict the chemical shift changes that should result from the addition of different amounts of copper (II) solution to the rumen fluid, and compare this with experimentally measured values.

The predicted chemical shift is given by

$$\delta_{pred} = \alpha_A\delta_A + \alpha_{HA}\delta_{HA} + \alpha_{CuAx}\delta_{CuAx} \quad (1)$$

where α_{pred} is the observed chemical shift: α_A, α_{HA}, α_{CuAx} the fractions of acetate, acetic acid and all different copper acetate complexes, respectively; and δ_A, δ_{HA}, δ_{CuAx} the chemical shifts of acetate, acetic acid and a composite of all the different copper acetate complexes, respectively. Results from the model closely fit the information obtained from the experiment. Figure 8 shows the fit. This suggests that the computer model adequately describes the speciation of Cu(II) with respect to LMM in the rumen fluid.

However, in order to stress the model further, another study was undertaken where varying amounts of lysine were also added to the rumen fluid as an exogenous ligand. Figure 9 illustrates changes in chemical shift of methyl protons of acetate as a function of copper(II) concentration as different amount of lysine are added. The computer model predicts that the addition of 0.8 m**M** lysine would not alter the speciation significantly due to the low concentration of the added lysine compared with the concentration of other ligandspresent. When actual ^{1}H NMR experiments were conducted this was also the case. The model closely predicts the observed changes in chemical shift. Small deviations are the result of experimental errors in the NMR, and show no systematic trend away from the predicted line.

The computer model predicts a further change in the speciation picture when 4.0 mM lysine is added. With this higher concentration of lysine, and considering the strength of the Cu-lysine complexes, lysine should bind more of the Cu(II). This reduces the amount

of Cu-acetate complexes, and hence a reduction in the chemical shift of the acetate is predicted. However, when these predictions are compared to the data obtained from an actual ^{1}H NMR experiments, some discrepancy is noted between the model and the experimental values.

When 20 mM of lysine were added, the model predicts that the bulk of the copper will now be present as copper lysine complexes. The effect on the acetate chemical shift is similar to that at 4.0 mM added lysine, but more pronounced. However, experimental data shows little change in the chemical shift of acetate with the increasing levels of copper. The discrepancy between model and experiment is now pronounced.

In summary: this model does not adequately explain the speciation at elevated levels of added lysine. The model predicts a lower amount of Cu-acetate complexes than is actually observed.

Several reasons could possibly account for this discrepancy. It could be a result of using values for the metal concentrations that do not accurately reflect those appearing in the model. To explore this possibility, the actual levels of metals in the fluid were measured using Inductively Coupled Plasma Atomic Emission [25]. Results displayed only minor differences from metal concentration values appearing in the literature. Using the experimentally derived metal levels in the simulation did not appreciably alter the results obtained, having a minimal effect on the predicted chemical shifts.

The discrepancy might also be due to an interaction of lysine with some other species in the rumen liquor, one not included in the model. However, we have previously performed similar NMR experiments in the simulated rumen fluid, which do not

contain the hypothesized "other species" [26]. Results were identical to those observed in authentic rumen fluid.

We therefore concluded that the problem lay with the model, probably with a lack of knowledge of all possible complexes that could form. In particular, the possibility of formation of mixed ligand complexes was explored since formation of mixed ligands are very common in biofluids [24]. The formation of low molar mass complexes in biofluids results from multimetal-multiligand equilibria involving exchangeable metal ions, amino acids and other biological ligands. Because potential bioligands in rumen fluid exceed Cu(II) ions in number and quantity, the possibility of the formation of Cu(II) mixed ligand complexes will be very high since the Cu(II) ion has been found readily to form mixed ligand complexes [24]. Probable mixed ligand complexes considered in this context include acetopropionatocopper(II), acetodiaminocopper(II) and acetolysinatocopper(II) (Figure 10).

All three mixed ligand complexes considered contain acetate, since it is the most abundant ligand in the rumen. Propionate is the second most abundant VFA in the rumen and share similaritires with acetate in the way in which they it bonds to Cu. Since Cu(II) ion is a borderline metal ion according to Pearson's Hard Soft Acid Base (HSAB) concept, it has preference for nitrogen-based ligands. Thus ammonia, although present in rumen fluid in relatively small amounts, was considered as another candidate for possible mixed-ligand complexation. The amino acid lysine is known to form stable complexes with Cu(II) with coordination taking place via the α-amino and the carboxylate group so as to form a five membered chelate ring. Since Cu(II) has six coordination sites, the other four remaining sites would be available for coordination

by other ligands in the rumen. Thus, mixed-ligand complexation involving lysine and acetate was also considered. Also, it was the introduction of lysine as an exogenous ligand that led to the observed discrepancy between prediction and experiment.

The formation constants for these mixed ligand species were estimated from their 1:1 copper complex formation constants; values are shown in Table 5. These complexes were introduced into the computer model one at a time. The introduction of acetolysinatocopper(II) into the model resulted in an excellent fit to the experimental data, as shown in Figure 11. Inclusion of the other two mixed-ligand complexes, either by themselves or together with acetolysinatocopper(II), did not result in any improvement to the fit.

It appears that as lysine is introduced into the rumen fluid, it is unable to strip copper completely off the acetate, but rather forms a mixed-ligand complex with it. This complex, previously unaccounted for in the computer model, also contributes to overall acetate chemical shift, accounting for the discrepancies observed between the experimental data and the model.

Similar experiments were conducted with actual rumen fluid. The results obtained (Figure 12) are similar to those obtained for simulated rumen fluid above. Again, the inclusion of acetolysinatocopper(II) results in excellent agreement between experimental results and simulation.

Computer simulations involving copper glycine and copper methionine were also validated with ^{1}H NMR experiments, the monitored parameter being the chemical shift of the methyl protons of acetate as before [6, 25]. Similar to the copper-lysine case

detailed above, there was an increase in discrepancy between the model-predicted chemical shifts and the observed chemical shifts as the concentration of the amino acid was increased. The implication is again that the simple model does not adequately explain the speciation. Mixed-ligand complex formation between copper, acetate and the relevant amino acid, when introduced into the computer models, again resulted in a much improved fit to the experimental data, as shown in Figures 13 and 14.

CONCLUSION

It is now generally recognized, when considering a metal ion in a biological system, that it is speciation rather than just the total concentration that influences bioavailability. Speciation modelling and measurement can help in understanding how different forms of supplementation achieve different degrees of bioavailability. Such models can be used as a convenient and cost effective tool in evaluating mal supplement bioavailabilities. However, as with any modeling technique, it is also important to validate such models by experimental means.

In this study computer simulation has been used to model the speciation of Cu(II) in rumen fluid, to help understand the distribution of copper in biofluids of ruminants with the ultimate aim of helping combat copper deficiency: a problem that confronts farmers daily. Comparison of the speciation of copper(II) glycine, copper(II) methionine, copper(II) lysine and copper(II) EDTA, which are being used as 'chelated' copper supplements for ruminants, showed that only copper histidine and copper EDTA do not degrade into other species when introduced into the bovine saliva and rumen fluid. For

other ligands, the study demonstrates that mere knowledge of the initial form of the supplement used is of limited value, as the many interactions in the rumen alter the chemical form ingested considerably. The form eventually absorbed will be significantly different from that ingested. Validation using ^{1}H NMR experiments disclosed that consideration of mixed-ligand complexation is essential for an adequate description of speciation in these systems.

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Table 1 Values of ligand concentrations used in rumen modeling

Ligand	Concentration (mM)
Acetate	84
Propionate	21
Butyrate	13
i-Butyrate	1
Valerate	0.7
i-Valerate	0.3
Phosphate	20
Carbonate (in all forms)	21
Ammonia (in all forms)	11

Table 2 Formation constant data used in rumen modeling ($\log \beta = [M_pL_qH_r]/[M]^p[L]^q[H]^r$).

System	p	q	r	log β
Acetic Acid	0	1	1	4.56
Copper Acetate	1	1	0	1.81
	1	2	0	2.83
	1	3	0	3.17
	1	4	0	3.23
Propanoic Acid	0	1	1	4.68
Copper Propanoate	1	1	0	1.70
	1	2	0	2.62
Butanoic Acid	0	1	1	4.62
Copper Butanoate	1	1	0	1.75
	1	2	0	2.70
iso-Butanoic Acid	0	1	1	4.62
Copper iso-Butanoate	1	1	0	1.75
(estimate)	1	2	0	2.70
Valeric Acid	0	1	1	4.62
Copper Valerate	1	1	0	1.75
(estimate)	1	2	0	2.70
Phosphoric Acid	0	1	1	11.40
	0	1	2	18.04
	0	1	3	19.99
Copper Phosphate	1	1	1	3.3
	1	1	2	1.3
Carbonic Acid	0	1	1	9.83
	0	1	2	15.41
Copper Carbonate	1	1	0	6.75
	1	2	0	9.92
	1	1	1	1.3
Ammonia	0	1	1	9.24
Copper Ammonia	1	1	0	4.12
	1	2	0	7.63
	1	3	0	10.51
	1	4	0	12.60

Table 3 Acid dissociation constants of amino acids and EDTA used in rumen modeling

The Glycine System	
pKa_1	2.450(2)
pKa_2	9.32(1)
The Histidine System	
pKa_1	1.90(1)
pKa_2	5.86(1)
pKa_3	8.73(2)
The Lysine System	
pKa_1	2.23(2)
pKa_2	8.92(2)
pKa_3	10.48(2)
The Methionine System	
pKa_1	2.42(2)
pKa_2	8.98(1)
The EDTA System	
pKa_1	1.6
pKa_2	2.05(5)
pKa_3	2.79
pKa_4	6.03(5)
pKa_5	10.02(7)

Table 4 Formation constants for copper complexes of exogenous ligands used in computer simulation studies ($\beta_{pqr} = [M_pL_qH_r]/[M]^p[L]^q[H]^r$).

p	q	r	$\log\beta_{pqr}$
The Copper (II) Glycine System			
1	1	0	8.17(3)
1	2	0	14.12(4)
1	2	-1	3.5(1)
1	1	1	11.78(4)
1	2	1	18.89(8)
The Copper (II) Histidine System			
1	1	0	9.88(1)
1	2	0	18.80(2)
1	1	1	13.93(8)
1	2	1	24.00(2)
1	2	2	27.49(6)
1	2	-1	11.85(3)
The Copper (II) Lysine System			
1	1	0	10.72(8)
1	1	1	18.01(2)
1	2	2	34.69(2)
1	2	1	25.39(7)
1	1	2	21.81(8)
1	2	0	15.30(5)
1	1	-1	.8(1)
The Copper (II) Methionine System			
1	1	0	7.66
1	2	0	14.60
The Copper (II) EDTA System			
1	1	0	18.53(8)
1	1	1	21.38
1	1	2	23.13

Table 5 Estimated formation constants of mixed-ligand complexes of copper, acetate and amino acids used in rumen simulation studies (log $\beta_{pqs} = [M_pL_qA_s]/[M]^p[L]^q[A]^s$) [8, 26-28].

System	p	q	s	log β_{pqs}
Copper-Acetate-Lysine	1	1	1	8.8
Copper-Acetate-Glycine	1	1	1	9.5
Copper-Acetate-Methionine	1	1	1	8.2

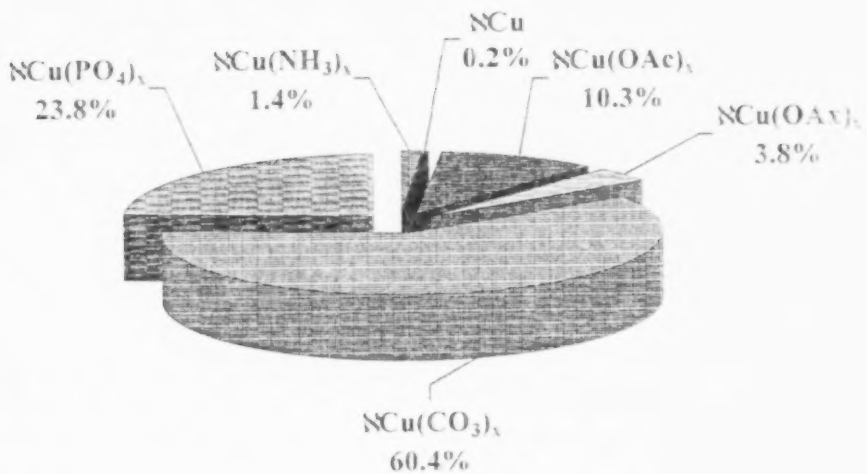

Figure 1 Distribution of copper with respect to the LMM ligands in rumen fluid at pH 6.3. Calculation were performed at an ionic strength of 0.15 M; temperature of 38 °C and Cu^{2+} concentration of 3.0×10^{-5} M. See Table 1 for concentrations of ligand.

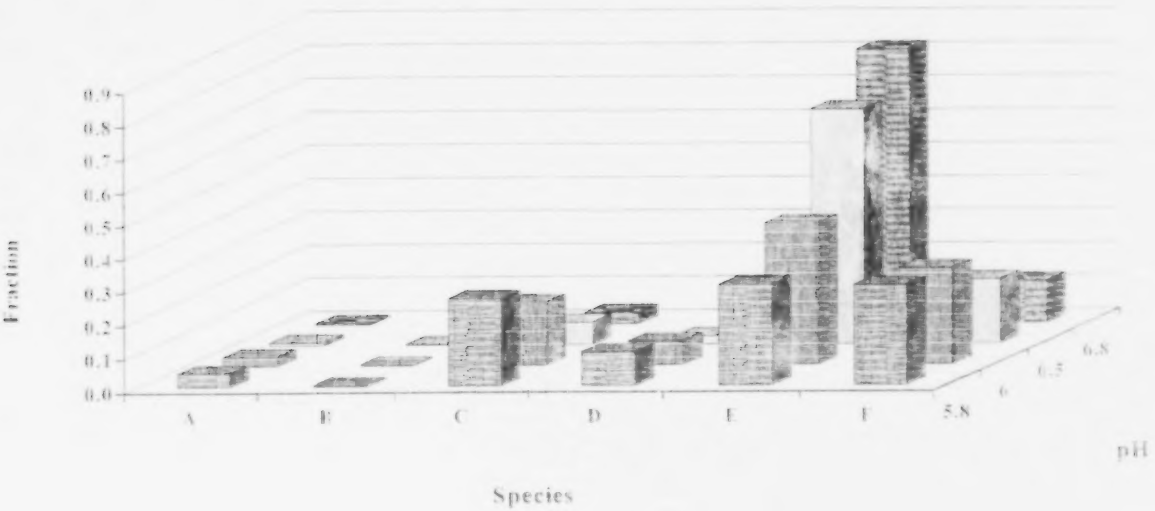

Figure 2 Distribution of copper with respect to the LMM ligands in rumen fluid as pH is varied between 5.8 and 6.8. Calculations were performed at an ionic strength of 0.15 M and Cu^{2+} concentration of 3.0×10^{-5} M. See Table 1 for concentrations of ligands. **A:** Copper ammonia complexes; **B:** Free Cu^{2+}; **C:** Copper acetate complexes; **D:** Copper VFA complexes; **E:** Copper carbonate complexes; **F:** Copper phosphate complexes.

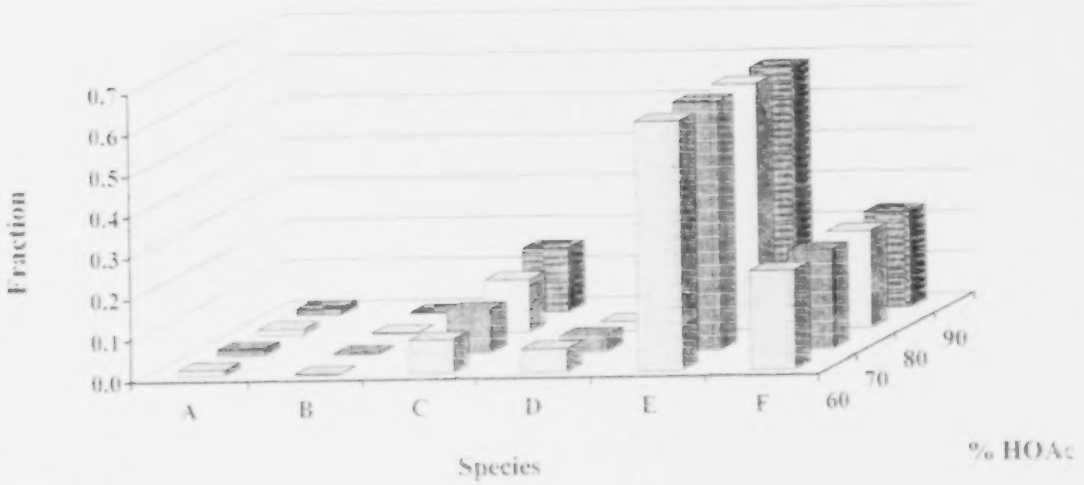

Figure 3 Distribution of copper with respect to the LMM ligands in rumen fluid as percentage of acetic acid is varied with respect to other VFAs. Calculations were performed at an ionic strength of 0.15 M and Cu^{2+} concentration of 3.0×10^{-5} M. See Table 1 for concentrations of ligands. **A:** Copper ammonia complexes: **B:** Free Cu^{2+}: **C:** Copper acetate complexes: **D:** Copper VFA complexes: **E:** Copper carbonate complexes: **F:** Copper phosphate complexes.

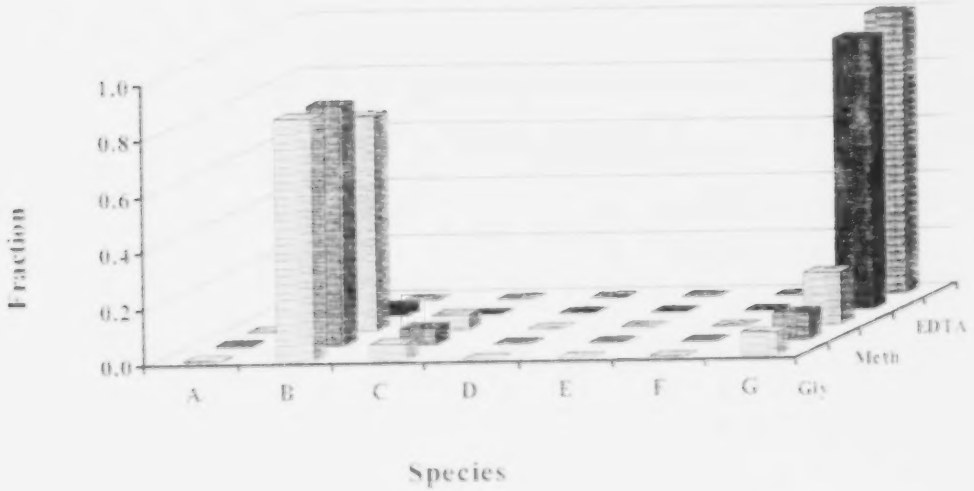

Figure 4 Comparison of speciation of copper in McDougall's solution of various copper supplements at physiological pH of 7.0 Calculations were performed at an ionic strength of 0.15 M, temperature of 38 °C and Cu^{2+} concentration of 3.0×10^{-5} M. See Table 1 for concentrations of ligands. **A:** Free Cu^{2+} : **B:** Copper carbonate complexes : **C:** Copper phosphate complexes : **D:** Copper (II) sulfate : **E:** Copper (II) chloride: **F:** Copper hydroxide complexes : **G:** Copper amino acid complexes

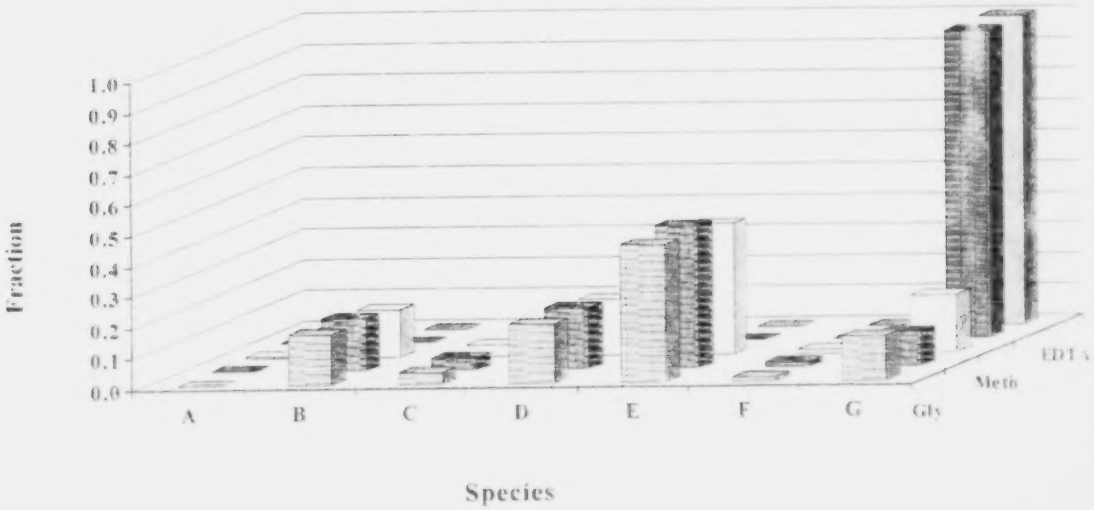

Figure 5 Comparison of speciation copper in rumen fluid of various copper supplements at ruminal pH of 6.3. Calculations were done at Ionic strength of 0.15 M; temperature of 38 °C and Cu^{2+} concentration of 3.0×10^{-5} M. See Table 1 for concentrations of ligands. **A:** Free Cu^{2+}; **B:** Copper acetate complexes; **C:** Copper VFA complexes; **D:** Copper phosphate complexes; **E:** Copper carbonate complexes; **F:** Copper ammonia complexes; **G:** Copper amino acid complexes.

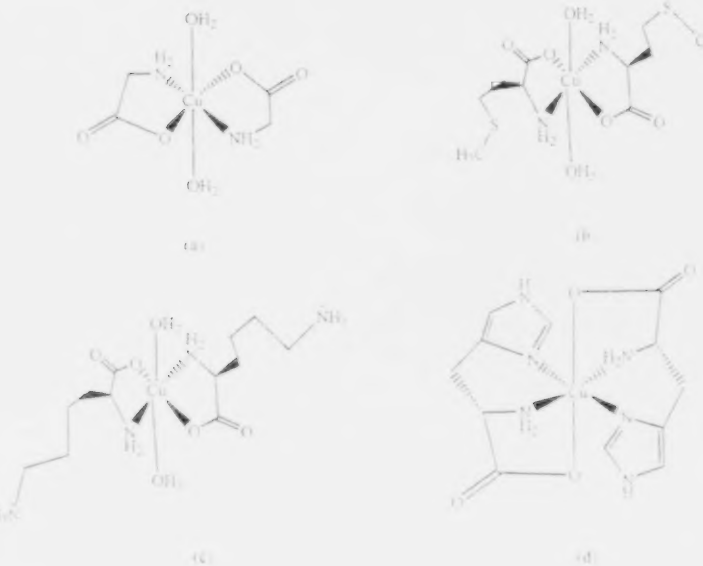

Figure 6 Structures of copper(II) biscomplexes of (a) glyine, (b) methionine, (c) lysine and (d) histidine

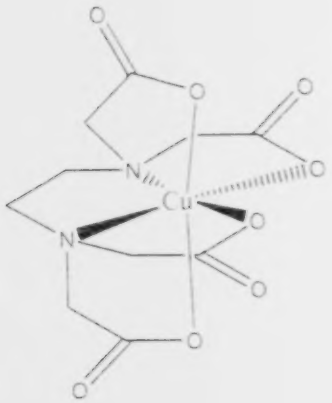

Figure 7 Structure of Copper(II) EDTA complex

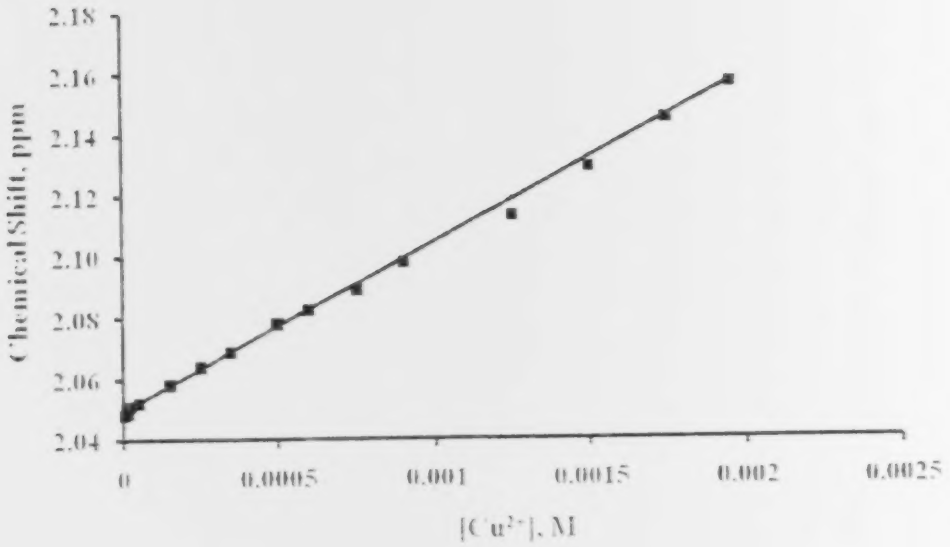

Figure 8 Chemical shift of acetate methyl protons in simulated rumen fluid as a function of copper(II) concentration. Curve shows predicted values and points (■) show experimental values.

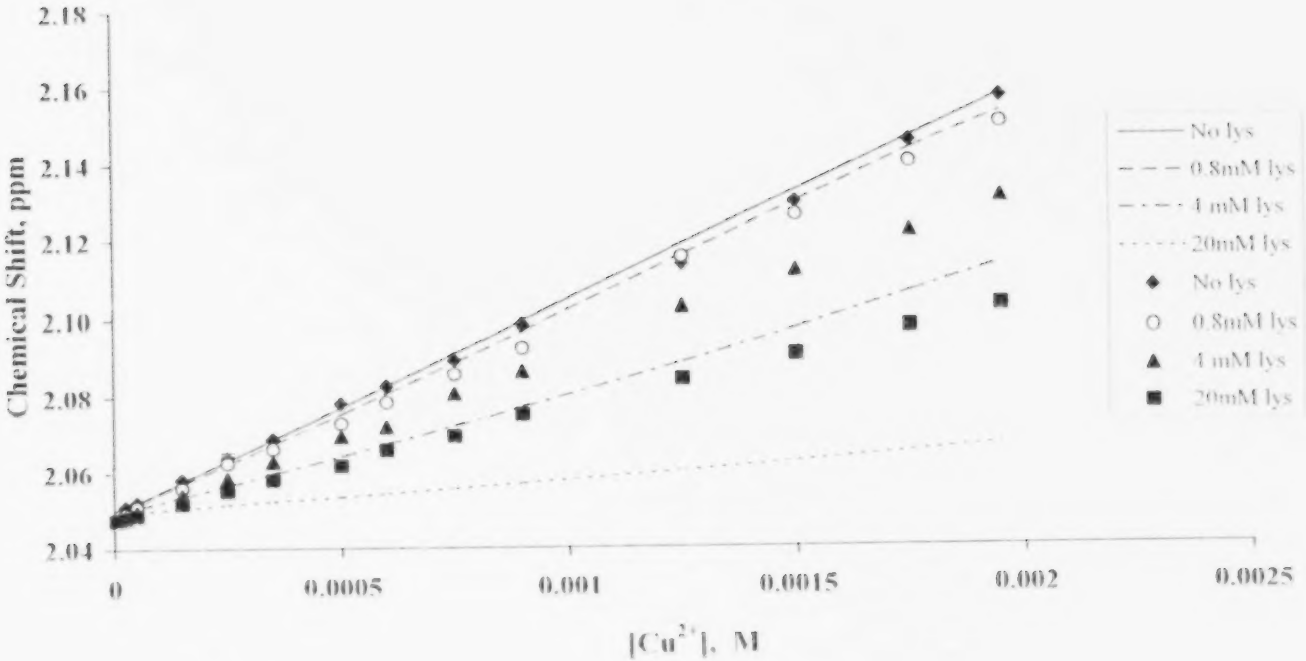

Figure 9 Changes in chemical shift of methyl protons of acetate with different amount of lysine as a function of copper(II) concentration in simulated rumen fluid. Points represent experimental data, while curves represent model predictions, using the previously developed model [6].

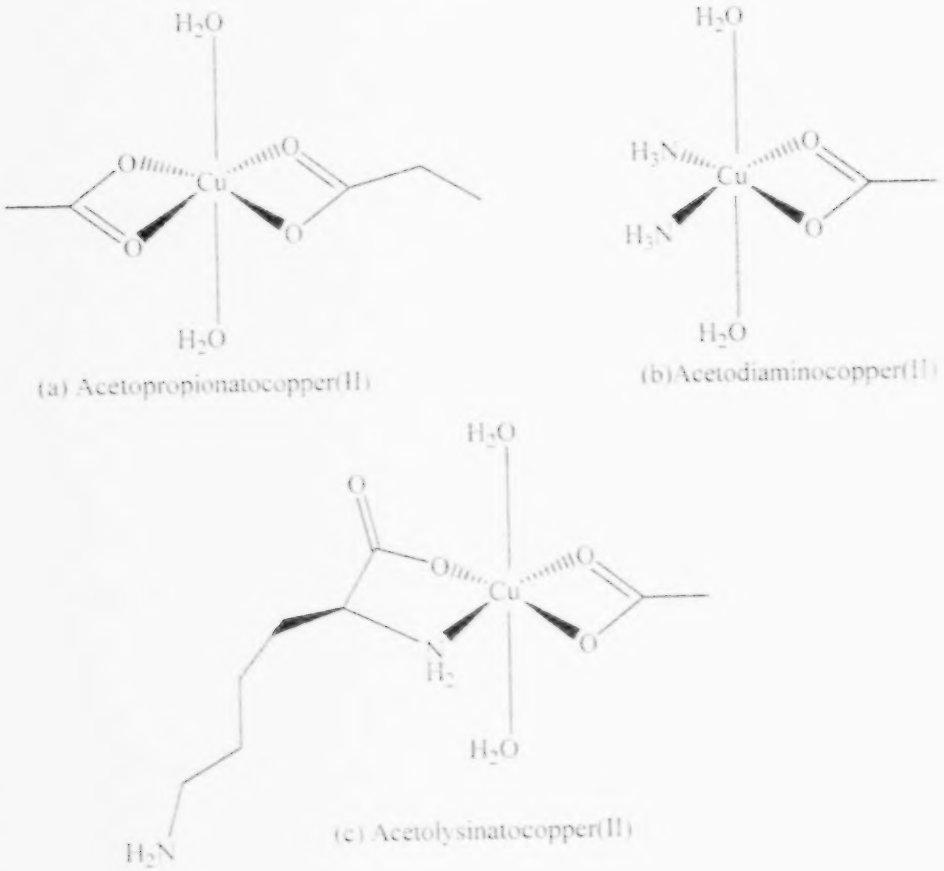

Figure 10 Possible Mixed ligand complexes of Cu(II) formed in rumen fluid

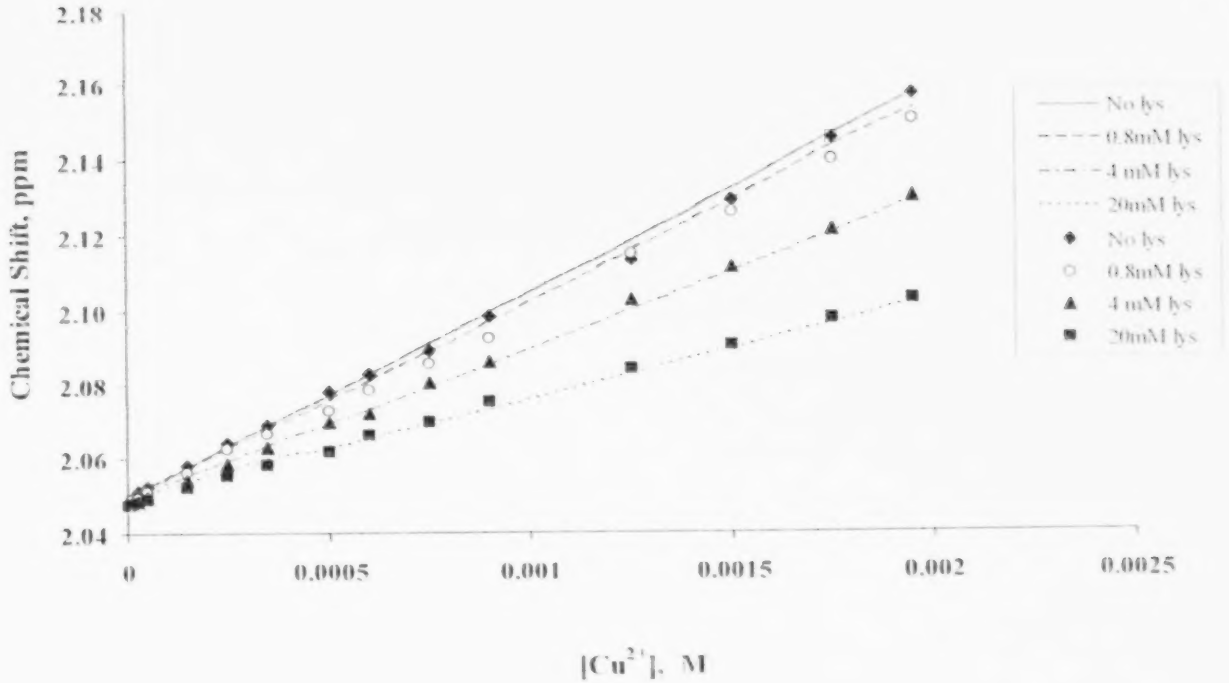

Figure 11 Changes in chemical shift of methyl protons of acetate with different amount of lysine as a function of copper(II) concentration in simulated rumen fluid. Points represent experimental data, while curves represent model predictions. Model now includes mixed ligand complexes.

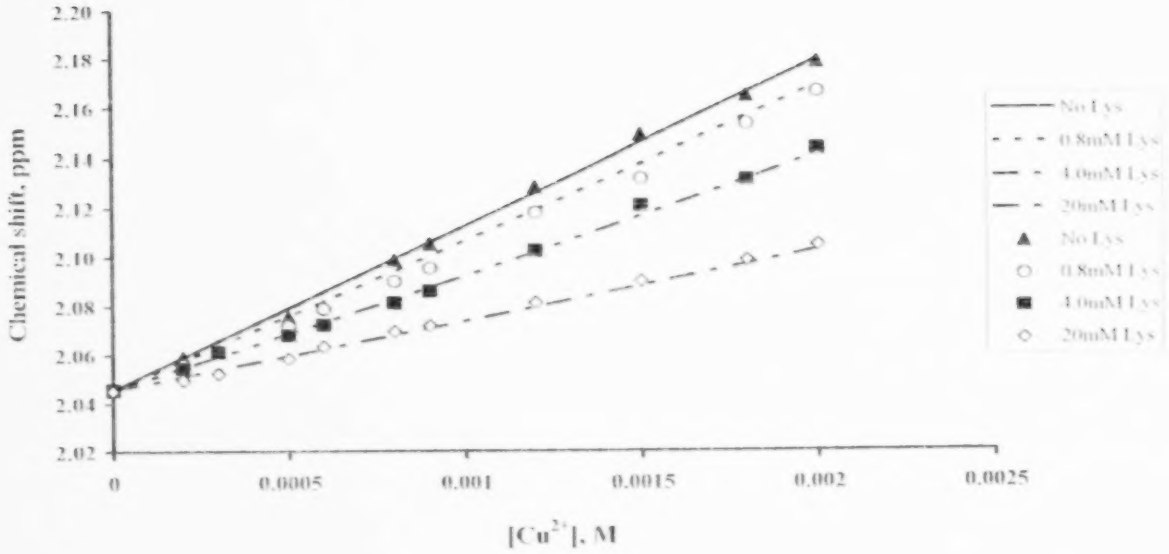

Figure 12 Changes in chemical shift of methyl protons of acetate with different amount of lysine in rumen fluid as a function of copper(II) concentration. Points represent experimental data while curves represent model predictions. Model includes mixed-ligand complexes.

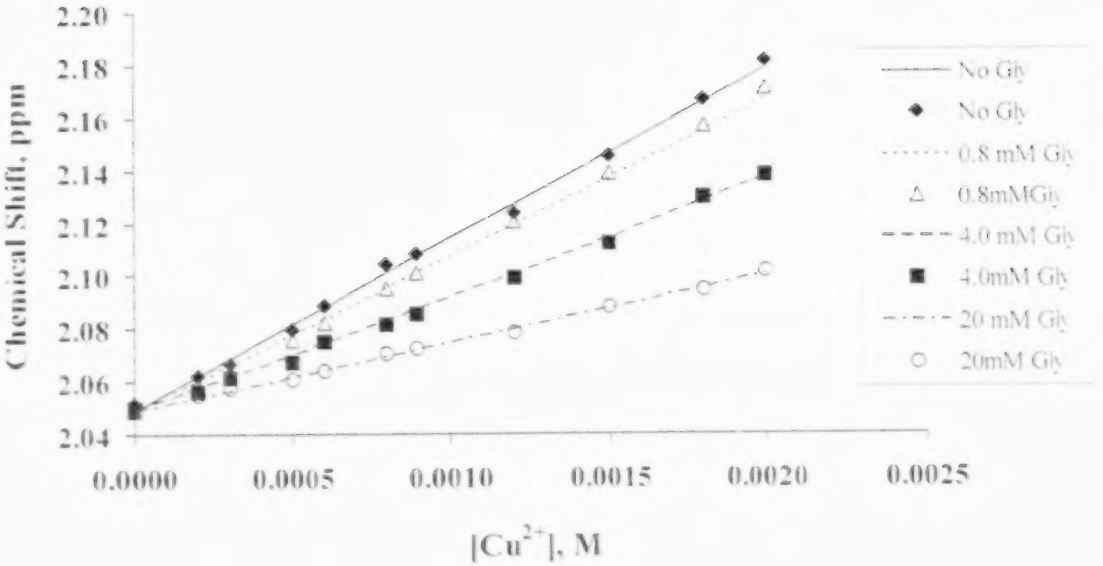

Figure 13 Changes in chemical shift of the acetate protons in actual rumen fluid as a function of added copper (II) concentration. Points represent experimental data while curves represent model predictions. Model includes mixed-ligand complexes.

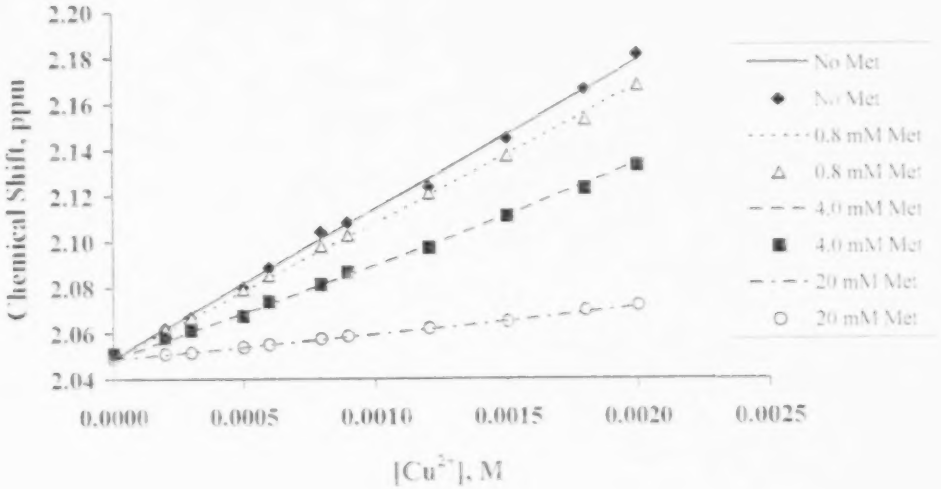

Figure 14 Changes in chemical shift of the acetate protons in actual rumen fluid as a function of added copper (II) concentration. Points represent experimental data while curves represent model predictions. Model includes mixed-ligand complexes.

Speciation Modeling Of Copper In Thiomolybdate - Contaminated Bovine Rumen Fluid

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Department of Chemistry
University of Saskatchewan

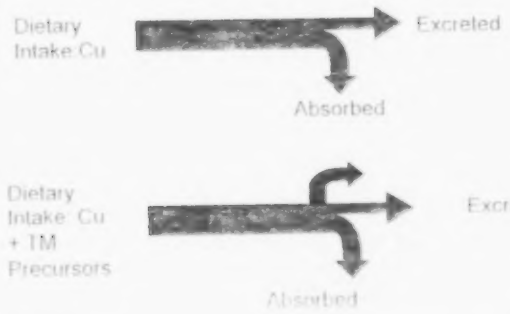
Outline

- Introduction
- Studies in our group
- Computer simulation in rumen fluid
- Validation of simulation
- Speciation of Cu supplements
- Speciation in the presence of thiomolybdates
- Conclusion and future studies

Introduction...1

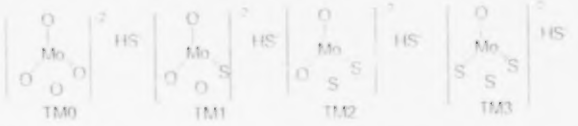
- Cu vital trace element in ruminants
  - required for metabolic activities
  - components of physiologically important metalloenzymes
- Cu deficiency → Distinctive pathologies → Economic losses
- Cu deficiency results from
  - low copper-in-diet (1°)
  - interference with Cu absorption in the animal due to Mo and S in food and/or water (2°)
    - Excessive uptake in formation of Thiomolybdates (TMo) in rumen

Introduction...2



Introduction...3

- Thiomolybdates – What are they?



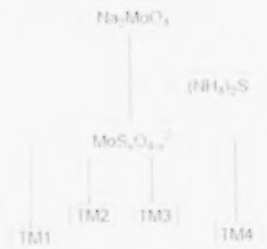
TM0- Molybdate
TM1- Monothiomolybdate
TM2- Dithiomolybdate
TM3- Trithiomolybdate
TM4- Tetrathiomolybdate

Introduction...4

- Synthesis of TMs was reported first in 1826 (Berzelius) and later in1922 (Debray)
- True composition & possible formation mechanism established in 1981(Muller & Diemann)
- Kinetics of formation 1980(Harmer & Sykes); 1982,1988(Brule *et al*); 1987(Clarke *et al*)

Introduction...5

- Lab Synthesis of TMS



- Replacement of O by S favored by
  - ↓ pH
  - ↑ S: Mo
  - ↑ temperatures
  - ↑ reaction times
- Nucleophile : HS^-
- In the rumen, S^0 formed by microbial reduction of SO_4^{2-}

Introduction.. 6

- Mo induced Cu deficiency established by Ferguson *et al* in 1938
- Cu/Mo/S interaction first reported in the 1950's by Dick *et al*
- Formation of bis(thiomolybdate) complexes between TMs and Cu(II) solution established (Clark & Laurie;1980,1982, 1986 & 1987)

Introduction...7

- Cu(II) and TM interaction (Price *et al*, 1987,1991)
  - initially forms stable but soluble substances
  - polymerizes to give stable and insoluble polymers

Studies in Reid group...1

- Computer Simulation, Potentiometry and ^{1}H NMR Speciation Studies of Cu(II) in
  - McDougall's solution (simulated bovine saliva)
  - bovine rumen fluid
- Results
  - Cu(II) Speciation similar in both inorganic and chelated forms
  - ^{1}H NMR experimental data compared well with computer simulation data of the saliva
  - Discrepancies observed for that of rumen

(M. A. Attaelmanan)

Studies in Reid group...2

- TMs synthesized and characterized using UV, ^{95}Mo NMR
- Solution Chemistry of interaction of Cu(II), TMs and some biological ligands
- Results
  - Formation of TMs kinetically controlled
  - Interaction of TMs with Cu(II) kinetically controlled
  - Once Cu(II) reacts with TMs neither S^{2-} nor EDTA can strip off Cu
  - Ternary complexation of BSA models, TMs and Cu(II) does occur

(F. E. Quaigrane)

Studies in Reid group...3

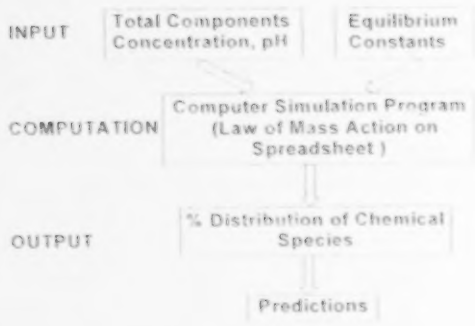
- Potentiometric and ^{1}H NMR determination of formation and acid dissociation constants of Cu(II)-mixed amino acid complexes
- Results
  - generally showed good agreement with literature values, where available
  - mixed ligand complexes formed often more stable than parent complexes

(M. Fiuza)

Objectives of Study

- Review computer modeling of Cu(II) speciation with respect to LMM ligands in bovine rumen
  - discrepancy between predicted and experimental results?
- Speciation modeling of 'chelated' Cu supplements in
  - bovine saliva
  - rumen fluid
- Speciation modeling of Cu(II) in the TM-contaminated rumen environment

Computer Simulation in Rumen Fluid... 1



Computer Simulation in Rumen Fluid... 2

- LMM Components of rumen fluid
- Volatile Fatty Acids

- NH$_4$
- Components of McDougall's solution (bovine saliva) - $MgSO_4$, $NaHCO_3$, NaCl, KCl, NaH_2PO_4, $CaCl_2$

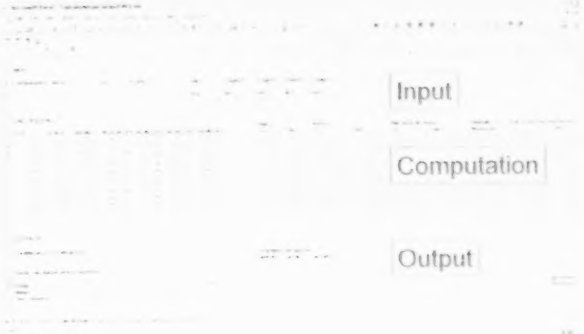
Computer Simulation in Rumen Fluid... 3

- Thermodynamic equilibrium constants obtained from
  - The IUPAC Stability Constants Database
  - NIST Stability Constants of Metal Complexes Database
- Formation constants critically evaluated
  - Temperature
  - Concentration
  - Background electrolyte
  - Ionic Strength
  - Computation
- Constants obtained at different conditions corrected by
  - Van't Hoff Equation
  - Davies Equation

Computer Simulation in Rumen Fluid…4

- Mass balance equations of Cu(II) and LMM of rumen
- Equations encoded in spreadsheet
- Iterative optimization algorithm used until all species have settled at equilibrium
- Back calculation of input parameters.

Computer Simulation in Rumen Fluid…5

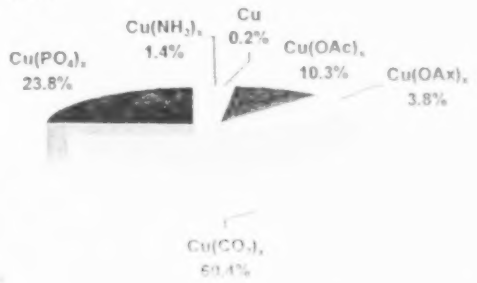


Computer Simulation in Rumen Fluid…6

- Iteration

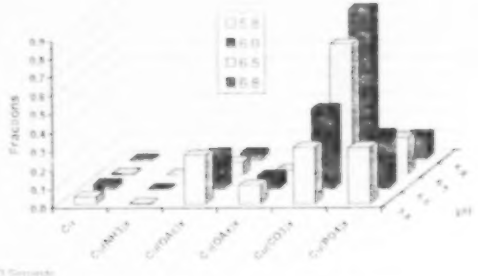
Computer Simulation in Rumen Fluid…7

- Cu distribution with respect to lmm ligands in rumen fluid at at pH of 6.3



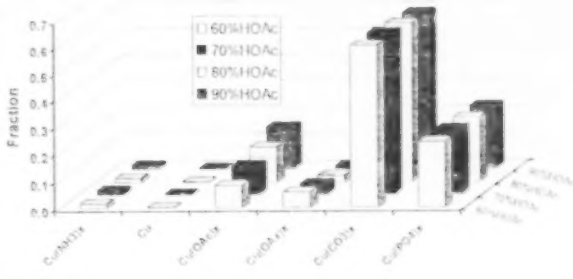
Computer Simulation in Rumen Fluid…8

- Temperature variation



Computer Simulation in Rumen Fluid…9

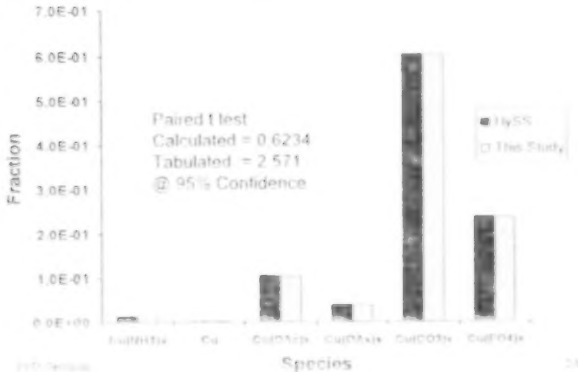
- VFA Concentration variation



Computer Simulation in Rumen Fluid…10

- Though internally consistent, results require verification and validation
- Results validated against
  - computer simulation program (HySS) and
  - experimental results (^{1}H NMR experiments on rumen fluid)

Computer Simulation in Rumen Fluid…8



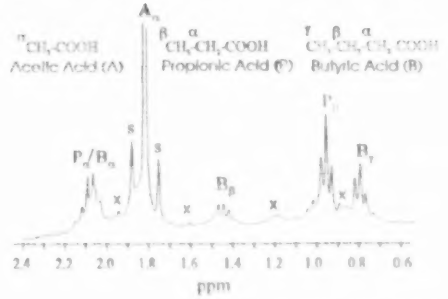
Validation of Computer Simulation…1

- Collection of Rumen fluid
- Rumen fluid
  - transported to lab in flask
  - ultracentrifuged
  - ultrafiltered

Validation of Computer Simulation…2

- NMR Sample Preparation
  - DSS added to ultrafiltered rumen fluid as chemical shift reference
  - Spectra were acquired at 500.13 MHz using the binomial composite '1-3-3-1' sequence for water suppression
  - also suppressed macromolecular resonances

Validation of Computer Simulation…3



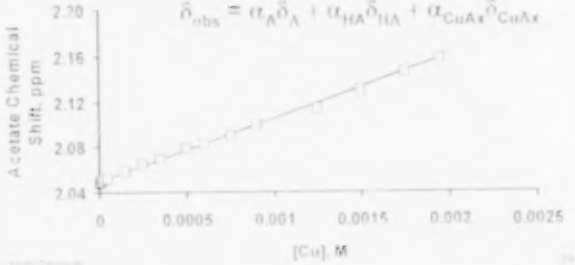
(M. A. Attaelmanan)

Validation of Computer Simulation…4

- Validation involves the comparison of predicted and experimentally determined values of some parameter.
- The parameter employed here is the ^{1}H NMR chemical shift of the acetate -CH_3 protons.
- The chemical shift of acetate was calculated with varying levels of Cu(II) concentration added to the solution.

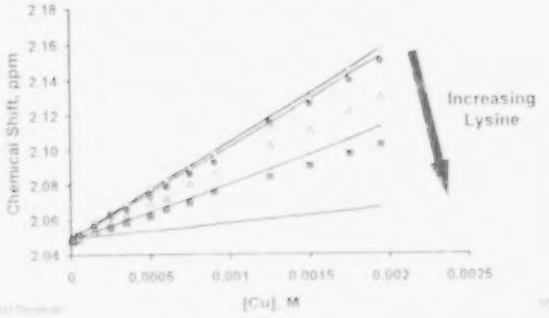
Validation of Computer Simulation...5

$$\delta_{obs} = \alpha_A\delta_A + \alpha_{HA}\delta_{HA} + \alpha_{CuAx}\delta_{CuAx}$$



Validation of Computer Simulation...6

- Now, add excess Lysine as well... oops!



Validation of Computer Simulation...7

- Possible reasons for discrepancy
  - Inaccurate values for the metal concentrations
  - Interaction of lysine with some species in the liquor
  - Ionic strength effects
  - Formation of colloidal complexes
- Possibility of formation of mixed-ligand complexes explored

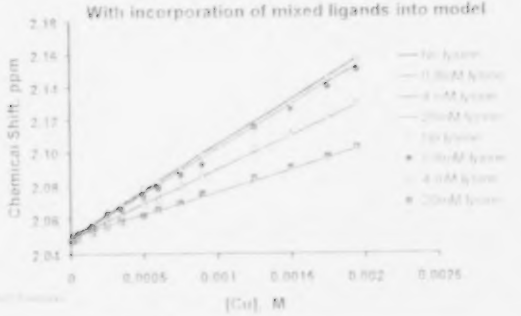
Validation of Computer Simulation...8

- Possible Mixed ligand complexes considered

Validation of Computer Simulation...9

- Changes in chemical shift of $-CH_3$ of acetate with different amount of lysine as function of Cu(II) concentration

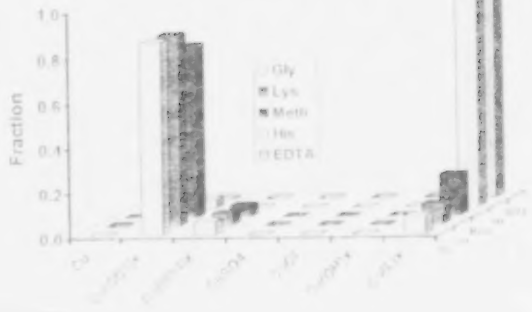
With incorporation of mixed ligands into model



Speciation of Cu Supplements in Bovine Saliva...1

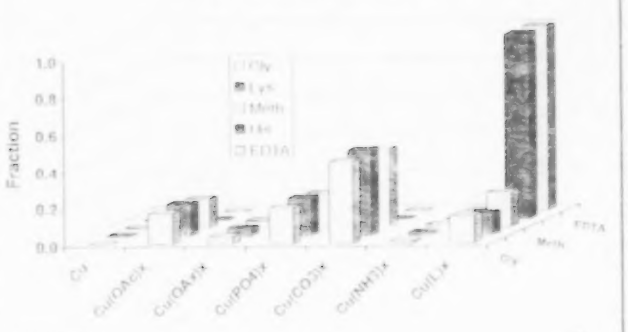
- Copper supplements: Cu Glycine, Cu Lysine, Cu Histidine, Cu Methionine and Cu EDTA
- Components of McDougall's Solution (bovine saliva)
  - $NaHCO_3$, Na_2HPO_4, KCl, NaCl, $MgSO_4$, $CaCl_2$
- Conditions
  - pH 7.0, Temperature 38 °C, Ionic Strength 0.15 M

Speciation of Cu Supplements in Bovine Saliva...2



Speciation of Cu Supplements in Bovine Saliva...3

Speciation of Cu Supplements in Bovine rumen fluid



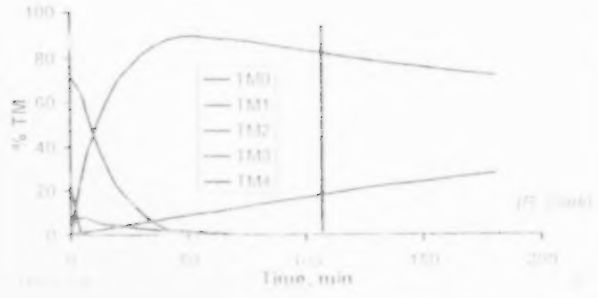
Speciation of Cu(II) in the presence of TMs

- Objective
  - Speciation Modeling of Cu(II) with respect to LMM ligands in the rumen ***in the presence of TMs***
- Challenges
  - Integrating
    - Cu(II) Speciation in rumen – ***thermodynamically controlled***

    with
    - Formation of TMs and Interaction with Cu – ***kinetically controlled***
  - Kinetic data available of variable quality

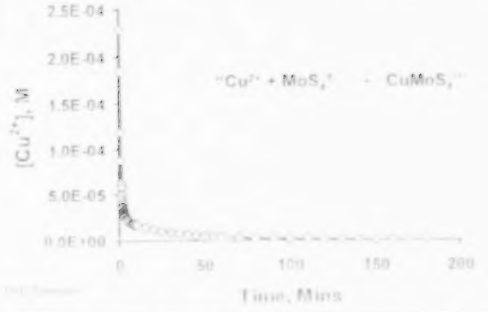
Thiomolybdates Formation

- Formation of TMs Kinetically controlled



Thiomolybdates and Cu(II)

- Interaction of Cu(II) with TMs also Kinetically controlled
- Kinetic study of Cu-TM4 interaction using Cu(II) ISE



Computer Simulation of the Formation of Thiomolybdates…1

- Behaviour described by a system of differential equations

$$\frac{d[TM0]}{dt} = k_1[TM0] - k_2[TM1]$$

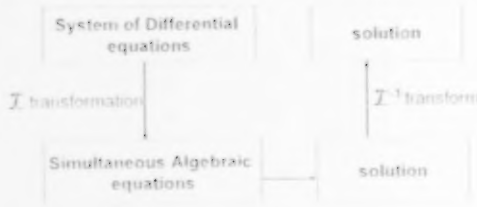
$$\frac{d[TM1]}{dt} = k_1[TM0] - (k_2 + k_3)[TM1] + k_4[TM2]$$

$$\frac{d[TM2]}{dt} = k_3[TM1] - k_4[TM2]$$

- To determine the system response with respect to time, the differential equations have to be solved

Computer Simulation of the Formation of Thiomolybdates…2

- Algebraic method using Laplace Transform



Computer Simulation of the Formation of Thiomolybdates…3

$$[TM0]_0 = a;\ [TM1]_0 = 0;\ [TM2]_0 = 0$$

$$[TM0] = a\left[\frac{k_1k_4 - k_3(r_1 - k_2)}{r_1(r_1 - r_2)}e^{r_1 t} - \frac{k_1k_4 - k_3(r_2 - k_2)}{r_2(r_1 - r_2)}e^{r_2 t}\right]$$

$$[TM1] = ak_1\left[\frac{k_4 - r_1}{r_1(r_1 - r_2)}e^{r_1 t} - \frac{k_4 - r_2}{r_2(r_1 - r_2)}e^{r_2 t}\right]$$

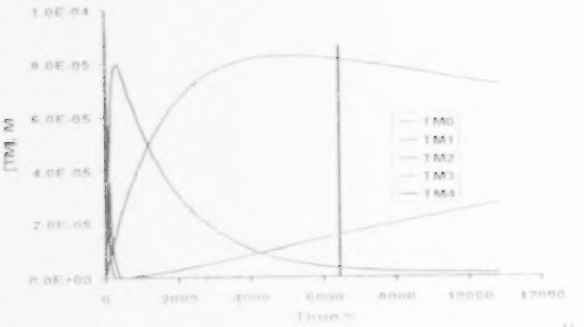
$$[TM2] = ak_1k_3\left[\frac{1}{r_1(r_1 - r_2)}e^{r_1 t} - \frac{1}{r_2(r_1 - r_2)}e^{r_2 t}\right]$$

where

$$r_1(r_2) = -\frac{k_1k_4 + k_2k_3 + k_3k_4}{2} \pm \frac{\sqrt{(k_1k_4 + k_2k_3 + k_3k_4)^2 - 4k_1k_3k_4}}{2}$$

- Implemented using Maple

Computer Simulation of the Formation of Thiomolybdates…4



Computer Simulation of Formation Cu-TM4…1

- TM4 produced at each time is reacted with Cu^{2+} available

$$TM4 + Cu^{2+} \xrightarrow{k} CuTM4$$

$[TM]_0 = a \quad [Cu^{2+}] = b$

$[CuTM4] = 0$

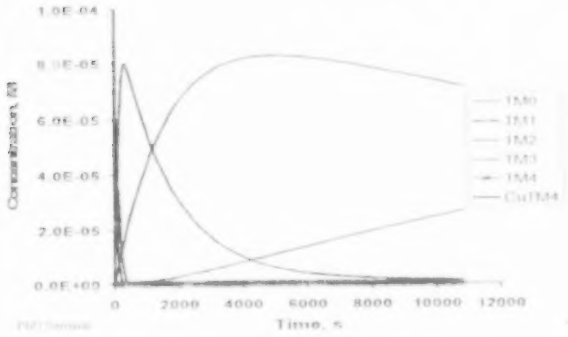
$$[TM4]_t = (b-a)\left|\frac{b}{a}e^{(b-a)kt} - 1\right|^{-1}$$

$$[Cu^{2+}]_t = (a-b)\left|\frac{a}{b}e^{(a-b)kt} - 1\right|^{-1}$$

$$[CuTM4]_t = a\left|\frac{a}{be^{(b-a)kt} - a} - 1\right|$$

Computer Simulation of Formation Cu-TM4…2

- Inclusion of the formation of CuTM4

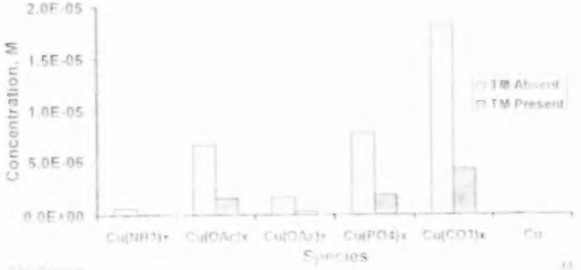


Integrating Cu/TM Kinetics with Thermodynamic Speciation Model

- Two Spreadsheets developed
  - Cu/TM kinetics
  - Cu Speciation in rumen fluid
- Cu/TM kinetics run for fixed time increments
- Equilibrium model run after each increment
- Done for about 107 mins (mean residence time of S^{2-} in the rumen)

Speciation of Cu(II) in the presence of TMs

- Effect of the of TM on Cu bioavailability



Conclusion and Future Work

- Conclusion
  - Effectively modelled Cu(II) speciation in rumen fluid contaminated with TMs
- Future work
  - Inclusion of the formation of CuTM3
  - Modeling of 'chelated' Cu supplements in the presence of TMs
  - Reliable kinetic data (R. Clark)
  - Spectroscopic studies of structure and stoichiometry of Cu-TM complexes (D. Slamova)
  - Influence of diet on Cu Speciation in rumen fluid in the presence of TMs

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- Past and present group members (L-R)

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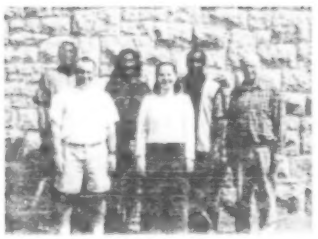
  R. Clark
  M. Burdu
  D. Slamova
  (self)
  Dr. S. Reid



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